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Diagnosis of Viral Respiratory Infections in the 1980s

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The past decade has seen major advances in laboratory diagnosis of infections caused by respiratory viruses. Two major factors account for much of the progress: the introduction of immunofluorescent and other immunologic techniques for rapid viral diagnosis and the availability of high quality commercial reagents needed for viral isolation and serologic testing. Because of these improvements, it is now possible for many hospitals to have access to viral diagnostic facilities and in some cases to have a result returned the same day!

Considerable time, effort, and cost are spent on each specimen submitted to a viral laboratory. For optimal efficiency, it is best for the laboratory to focus on those cases in which a definitive diagnosis may influence patient management. For the most part, this means attempts at viral diagnosis should be limited to those patients requiring hospitalization.

Specific diagnosis influences patient care in a number of ways. For some viral illnesses, such as encephalitis and neonatal infection caused by herpes simplex virus or infections in the immunocompromised host caused by varicella-zoster virus, antiviral chemotherapies have proven value.[14, 15] For these infections a definitive diagnosis is important to guide therapy. Other less obvious but important benefits of specific viral diagnosis include stopping unnecessary medications, such as antibiotics, and cancelling unneeded laboratory studies once a diagnosis is established. In addition, proper measures can be instituted to minimize spread of nosocomial infection. Perhaps most importantly, once a diagnosis is established the patient can be told specifically what illness he has and an accurate prognosis can then be given.

A large number of viruses cause respiratory infections. In fact, almost all viruses can, on occasion, infect the upper or lower airway. However, certain viruses are common causes of respiratory infection (Table 1).

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Table 1. *Common Viral Causes of Respiratory Infection*

INFECTION	COMMON VIRAL CAUSES
Upper Respiratory Tract Infections	
Common cold	Rhinoviruses, coronaviruses, respiratory syncytial virus, parainfluenza viruses
Tonsillitis, pharyngitis	Adenoviruses, Epstein-Barr virus, influenza viruses, parainfluenza viruses
Lower Respiratory Tract Infections	
Croup	Parainfluenza viruses, respiratory syncytial virus
Tracheobronchitis	Respiratory syncytial virus, parainfluenza viruses
Bronchiolitis	Respiratory syncytial virus, parainfluenza viruses
Pneumonia	
Children	Respiratory syncytial virus, parainfluenza viruses, adenoviruses, influenza viruses, measles
Adults	Influenza viruses, adenoviruses, varicella, measles
Immunocompromised patients (all ages)	Cytomegalovirus, varicella-zoster virus, herpes simplex virus

Attempts at viral diagnosis can be considered under several categories: viral isolation, serologic testing, direct detection of viruses by rapid techniques, and histologic or cytologic diagnosis.

For best results, the laboratory should be informed of the clinical syndrome and age of the patient. This information guides decisions as to which cell lines to inoculate, which viruses to seek by rapid techniques, and which antigens to include in serologic assays.

VIRAL ISOLATION

Specimens should be collected as early in the illness as possible since viral shedding is greatest at that time. A throat, nasopharyngeal swab, or nasopharyngeal aspirate is usually adequate for most respiratory infections. Sputum samples can be collected for isolation when there is evidence of lower respiratory disease. Tissue samples obtained, such as lung or bronchial brushings, are excellent specimens for viral isolation. When swabs are collected, cotton, dacron, or rayon swabs can be used. Alginate swabs inhibit growth of certain viruses and are not recommended.[5] Swabs or tissue samples do best if kept moist in viral transport medium. Body fluids such as sputum, pleural fluid, or bronchial washings do not require special additives and can be sent to the laboratory as collected.

Several systems are available for isolation of respiratory viruses, including tissue culture, egg, and laboratory animal inoculations. Of these, tissue culture is the most practical and the most commonly used.

Tissue Culture

The cell types include primary monkey kidney (usually rhesus), human diploid cells (often human embryonic lung cells), primary human embryonic kidney cells (HEK), and continuous human epithelial cells (HEP-2). The types of viruses isolated in these cells are shown in Table 2. These cells can

Table 2. *Optimal Cell Types for Viral Isolation**

VIRUS	PREFERRED CELL TYPES
Influenza	RMK or CMK
Parainfluenza (types 1–4)	RMK
Respiratory syncytial virus	HEP-2
Adenoviruses	HEK
Rhinoviruses	HEK, HDCS
Coronaviruses	Human tracheal organ cultures
Measles	HEK, RMK
Varicella	HDCS, HEK
Cytomegalovirus	HDCS
Herpes simplex virus	HEK, HDCS

*All cell types, except tracheal organ cultures, are available commercially from large biologic supply laboratories in North America. Abbreviations: RMK = rhesus monkey kidney; CMK = cynomolgus monkey kidney; HEP-2 = human epidermoid carcinoma cells; HEK = human embryonic kidney; and HDCS = human diploid cell strain (embryonic lung fibroblasts, foreskin fibroblasts, tonsillar fibroblasts).

be obtained commercially as monolayer cultures in either tubes or flasks. Human diploid cell strains and epithelial (HEP-2) cells can be maintained by serial passage in the laboratory, while primary cells must be purchased weekly. Cells are maintained in medium (Eagle's Minimal Essential Medium) supplemented with fetal calf serum (2 per cent) and antibiotics. Penicillin or vancomycin, gentamicin, and amphotericin B are useful combinations of antibiotics. Prior to inoculation, tissue culture cells should be maintained free of antibiotics to ensure detection of bacterial or fungal contaminants. On occasion, testing should also be performed to detect mycoplasmal contaminants. Reagents for this are available from commercial sources.

When specimens arrive in transport medium, this fluid is used to inoculate cell cultures. If sputum is too viscous to be inoculated directly, it can be diluted with transport medium and pipetted repeatedly to break up mucus. Tissue samples are homogenized in transport medium. The homogenate is clarified by low speed centrifugation and the supernatant used to inoculate cell cultures. Prior to inoculation, specimens are treated with antibiotics for approximately 20 minutes at room temperature. In our laboratory, 0.2 ml of each specimen is inoculated onto 3 or 4 cell types. During the winter months, respiratory specimens from children under 2 years of age are inoculated onto HEP-2 cells to detect respiratory syncytial virus. Specimens from all age groups are placed onto primary rhesus monkey kidney, human embryonic lung, and human embryonic kidney cells.

The time required to detect viral growth from most respiratory specimens is 5 to 7 days. Detection methods for the common respiratory pathogens include observing cultures for cytopathology (CPE) and determining whether red blood cells (usually guinea pig) adhere (hemadsorb) to the surface of inoculated cultures. An accurate presumptive diagnosis of the type of virus isolated can be made by noting the cell type on which CPE develops, the morphology of the CPE, and determining whether the infected cells are hemadsorption positive or negative (Table 3). A definitive identification can

Table 3. *Identification of Common Respiratory Pathogens*[*]

VIRUS	PRESUMPTIVE IDENTIFICATION			DEFINITIVE IDENTIFICATION
	Cytopathology	Cell Type	Guinea Pig RBC Adsorption	
RSV	Syncytium	HEP-2	Negative	FA†
Influenza A, B	Vacuolation and lysis	RMK	Positive	HI for subtyping‡
Parainfluenza 1	Minimal changes	RMK	Positive	FA,† Nt
Parainfluenza 2	Syncytium		Positive	FA,† Nt
Parainfluenza 3	Minimal changes		Positive	FA,† Nt
Adenoviruses	Granular, rounding	HEK	Negative	FA† for group antigen Nt for individual type
CMV	Foci of swollen rounded cells	HDCS	Negative	Not necessary
HSV	Rounded, ballooned cells	HDCS	Negative	FA†

*Abbreviations: RSV = respiratory syncytial virus; CMV = cytomegalovirus; HSV = herpes simplex virus; HEP-2 = human epidermoid carcinoma cells; RMK = rhesus monkey kidney; HEK = human embryonic kidney; HDCS = human diploid cell strain; FA = immunofluorescence; HI = hemagglutination inhibition; Nt = neutralization; RBC = red blood cell.

†Reagents are commercially available from large biologic laboratories.

‡Performed by state laboratories. Reagents are supplied to state laboratories by the Centers for Disease Control.

then be performed using immunologic reagents that react specifically with the isolated virus (Table 3).

Egg Inoculation

This method is sometimes used as a sensitive system for isolating influenza A and B viruses. Egg incubation is performed at 37 C and at 40 to 70 per cent humidity. The clinical specimen is prepared as for tissue culture inoculation and introduced into the amniotic and allantoic cavities of 10 to 11 day old fertile chick eggs. After 48 to 72 hours the amniotic and allantoic fluids are harvested and tested for virus by hemagglutination. For routine viral culturing, primary rhesus monkey cells are adequate and more easily incorporated into laboratory routine than use of egg inoculation.[8]

Animal Inoculation

Use of laboratory animals for viral isolation is expensive and generally restricted to highly specialized laboratories for those specimens of unusual interest or importance.

SEROLOGIC TESTING

Serologic diagnosis of respiratory viral infections has limited application. The types of viruses that cause respiratory disease are so numerous that serologic testing that includes all or most antigens is impractical. In addition, for some viruses such as rhinoviruses, there is no group antigen shared by all types; therefore, each of the 90 or more immunotypes must be tested for separately. Because of these shortcomings, in our laboratory we emphasize

Table 4. *Serologic Tests for Respiratory Viruses*

ANTIGEN	SEROLOGIC ASSAY
Influenza A	CF,* HI*
Influenza B	CF,* HI*
Parainfluenza (types 1–3)	CF, HI†
RSV	CF‡
Measles	CF, HI
Adenoviruses	CF§
CMV	CF

*The complement fixation (CF) test for influenza A and B is type specific. For strain-specific diagnosis the HI test is recommended. Reagents for hemagglutination-inhibition (HI) should include currently circulating influenza viruses, which may not be commercially available.

†A heterotypic antibody response to parainfluenza may occur by both CF and HI testing, that is, infection with one serotype induces an antibody titer rise to a different serotype.

‡Many infants fail to mount a CF response following RSV infection.

§Not all infections result in serologic responses, especially in infants and children.

viral isolation and rapid viral diagnosis (see below) for respiratory specimens and usually reserve serologic testing for those agents that are difficult to isolate (such as measles or mycoplasma).

A wide variety of respiratory viral antigens are available commercially for serodiagnosis. Some of the commonly used serologic tests are listed in Table 4. Sera drawn during both the acute and convalescent stages of disease are required. The acute serum should be drawn as close as possible to the time of onset of illness and stored at 4 C until blood in the convalescent stage of disease is drawn 2 to 3 weeks later. The sera are then tested simultaneously.

IgM antibody detection methods are available for diagnosis of active infection following a variety of viral illnesses. These include diagnosis of hepatitis A, Epstein-Barr, rubella, and cytomegalovirus infections.[1, 2, 10, 13] IgM methods have not yet been applied to the routine diagnosis of respiratory virus illnesses. As methodology improves, particularly in the use of solid-phase microtiter assays in which wells are coated with anti-human IgM antibody, we are likely to see increased application of IgM detection for the diagnosis of respiratory viruses.

RAPID VIRAL IDENTIFICATION

Immunofluorescence

The technique used to collect specimens for rapid diagnosis by immunofluorescence is of primary importance, since an adequate number of intact epithelial cells is necessary. A nasopharyngeal (NP) aspirate is generally the best specimen. A nasopharyngeal swab is less useful. The aspirate is collected by inserting a suction catheter (#6 catheter for children) through the nose into the nasopharynx. After suction is applied, the secretions are collected into a lukens trap by aspirating transport medium through the catheter. This material is then centrifuged at 700 × g for 10 minutes, and the sedimented

Table 5. *Rapid Diagnosis of Respiratory Viral Infections by Indirect Immunofluorescence**

TISSUE CULTURE	IMMUNOFLUORESCENCE		
	Positive	Negative	Total
Positive	45	8	53
Negative	6	46	52
Total	51	54	105

*See text for detailed explanation.

cells are washed in phosphate buffered saline (PBS) 1 to 3 times to rid them of excess mucus. The supernatant is inoculated into routine tissue culture for viral isolation and results compared with those obtained by immunofluorescence. For immunofluorescence, the cells are resuspended in a small amount of PBS, dropped onto a clean glass slide, and air dried. Samples from biopsy or autopsy can be prepared as impression smears (touching the tissue to the glass slide) or by pressing tissue between two slides. Once air-dried, slides are fixed in cold (−20 C) acetone prior to staining.

Specific antisera for direct or indirect immunofluorescence are available from commercial sources. These include antisera for respiratory syncytial virus, influenza A and B, parainfluenza types 1 through 3, herpes simplex, and adenoviruses.

Interpreting stained specimens requires experience to distinguish viral from nonspecific fluorescence. Specimens containing mucus or inflammatory cells are often difficult to read because of background fluorescence, which can be reduced by use of 0.1 per cent Evans blue counterstain.

Table 5 lists the results in our laboratory of immunofluorescence for rapid identification of respiratory syncytial virus in 1980. Specimens were obtained from hospitalized children with bronchitis, bronchiolitis, or pneumonia. Results were often available within 2 to 3 hours of receiving a specimen. In 85 per cent of culture-positive samples, immunofluorescence was also positive, and in 88 per cent of culture-negative samples, immunofluorescence was negative. Six samples were immunofluorescent positive and culture negative. The

Table 6. *Correlation Between Direct Immunofluorescence and Viral Isolation**

VIRUS	ISOLATION POSITIVE		ISOLATION NEGATIVE		OVERALL AGREEMENT
	FA positive	FA negative	FA positive	FA negative	
Respiratory syncytial virus	611	7	41	1387	97.7%
Influenza A (H_3N_2)	274	10	17	2261	98.9%
Parainfluenza 1	120	10	11	4918	99.6%
Parainfluenza 2	57	3	11	1544	99.1%
Parainfluenza 3	211	11	34	5508	99.2%

*Adapted from Gardner, P. S., and McQuillin, J.: Rapid Virus Diagnosis. Application of Immunofluorescence. Edition 2. London, Butterworth, 1980. FA = immunofluorescence.

interpretation of this is difficult without serologic confirmation of infection. As noted by Gardner, specimens collected late in an illness may be positive by immunofluorescence but negative by routine viral isolation.[6]

Immunofluorescence is useful for rapid diagnosis of several viral causes of respiratory illness including those caused by respiratory syncytial, influenza, and parainfluenza viruses. The correlation between immunofluorescence and viral isolation can be as high as 99 per cent (Table 6).

Enzyme Immunoassays (EIA) and Radioimmunoassays (RIA)

A number of respiratory viruses have been detected in nasopharyngeal aspirates by EIA or RIA. These include influenza A virus, respiratory syncytial, parainfluenza 2, and adenoviruses.[3, 7, 11, 12] These assays are approximately as sensitive as immunofluorescence for rapid diagnosis of respiratory infections. In epidemics or for epidemiologic surveys they are more convenient than immunofluorescence for testing large numbers of samples. However, in most clinical laboratories only one or two requests for rapid viral diagnosis are made daily. Under these circumstances, EIA and RIA may be more tedious and require more technician time than immunofluorescence.

HISTOLOGIC OR CYTOLOGIC DIAGNOSIS

Histologic sections of lung tissue may suggest a specific etiology to a viral pneumonia.[9] Histologic examination, however, is both less specific and less sensitive than viral isolation for defining the cause of the pneumonia. In patients with cytomegalovirus pneumonitis, characteristic inclusions in alveolar lining cells, macrophages, and occasionally endothelial cells may develop. The inclusions are basophilic, predominantly intranuclear, and are associated with marked cytomegaly. The intranuclear inclusions are surrounded by a clear zone (Fig. 1), referred to as a Cowdry type A inclusion. The morphology of cells infected with cytomegalovirus can easily be distinguished from changes produced by herpes simplex or varicella-zoster virus. These latter two viruses, however, cannot be distinguished from one another by morphologic criteria. Changes produced by both viruses include ground glass nuclei filled with basophilic inclusion material and marginated chromatin forming a thickened nuclear membrane, typical eosinophilic Cowdry type A intranuclear inclusions, and multinucleated giant cells with intranuclear inclusions. Respiratory syncytial virus produces cytoplasmic inclusions in bronchial epithelium but does not produce intranuclear inclusions. Measles virus causes multinucleated epithelial cells containing both nuclear and cytoplasmic inclusions. Adenoviruses produce eosinophilic and basophilic intranuclear inclusions somewhat similar to those seen in herpes simplex or varicella-zoster infection. Adenovirus inclusions, however, are usually larger, less clearly defined, and often separated from the nuclear membrane by a characteristic bubbly zone. In addition, multinucleated giant cells do not develop following adenovirus infections.

Cytologic specimens prepared from sputum, bronchial washings, or bronchial brushings occasionally demonstrate characteristic viral inclusions.[4] If possible, the diagnosis based on cytologic examination should be confirmed by attempts at viral isolation.

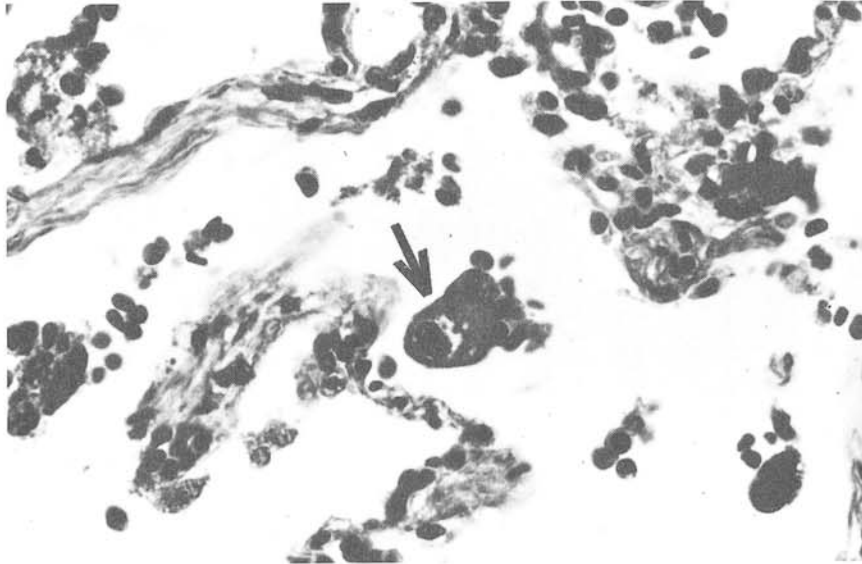

Figure 1. Cytomegalovirus pneumonitis. A typical Cowdry type A intranuclear inclusion is shown within a megalic cell (*arrow*) (500 × magnification).

CONCLUSIONS

The current trend in viral diagnosis of respiratory infections has been to emphasize methods for rapid diagnosis. Every effort should be made to include these techniques into the laboratory operations. However, they are not a substitute for routine viral isolation, which remains the "gold standard" by which all new methods are evaluated.

For laboratories just beginning in the area of diagnostic virology, attempts should first be directed at viral isolation using primary monkey kidney cells, human embryonic lung cells and, for patients under 2 years of age, HEP-2 cells. If possible, human embryonic kidney cells should also be used. In our laboratory we use one tube of each cell type and find this approach more cost-effective and efficient than duplicate tubes of each. When the laboratory is prepared to expand, rapid viral diagnosis should then be attempted. Our experience indicates that of the various approaches, immunofluorescence is most efficient. Serologic diagnosis, particularly for those agents that are difficult to cultivate, is the next priority. With these diagnostic tools, the laboratory is well equipped to handle the challenge of the 1980s.

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