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Efficiency of Monoclonal Antibodies to Detect Respiratory Virus Antigens in Clinical Specimens

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EFFICIENCY OF MONOCLONAL ANTIBODIES TO DETECT RESPIRATORY VIRUS ANTIGENS IN CLINICAL SPECIMENS

by

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ABSTRACT

The development of specific antiviral therapy has increased the need for rapid viral diagnosis and necessitates a better understanding of viral epidemiology. The purposes of this study were to determine the efficiency of immunofluorescent assay employing monoclonal antibodies from commercial sources to detect respiratory virus antigens in clinical specimens and to define the epidemiology of respiratory viruses in Bismarck, North Dakota.

Throat swab, nasopharyngeal swabs and nasopharyngeal washes were excellent specimens for studying viral epidemiology; virus isolation rates were 7.7%, 33.2%, and 39.7%, respectively. Yearly epidemics of respiratory syncytial virus and influenza were observed. In contrast, parainfluenza and adenovirus infections occurred endemically throughout the study period. Children less than 6 years of age and adults greater than 50 years of age had the highest viral morbidity. Respiratory syncytial and influenza were the most frequently isolated viruses.

Nasopharyngeal swab and nasopharyngeal wash specimens were evaluated for the ability to harvest ciliated epithelial cells from the upper respiratory tract. Overall, 8.7% of the specimens were unsuitable for immunofluorescent assay. The probability of specimen rejection was source- and patient age-dependent. The rejection rate for NPW specimens was more than twice that for NPS specimens and the

rejection rate for specimens from patients greater than 7 years of age was approximately 5 times higher than the rate for specimens obtained from patients less than 6 years of age.

Immunofluorescent assay was a sensitive and specific method for rapid diagnosis of respiratory viral infections in cell cultures. The Bartels indirect fluorescent antibody reagents exhibited acceptable levels of sensitivity (overall 84.6%, range 33.3 - 100%) and specificity (overall 83.3%, range 87.3 - 100%). The Whittaker direct fluorescent antibody reagents had poor sensitivity (overall 50%, range 18.2 - 94.1%) and high specificity (overall 91.5%, range 93.8 - 100%). The highly efficient Bartels reagents for respiratory syncytial virus and influenza A virus could be used as acceptable alternatives to culture for these viruses. The specificity of the influenza B, parainfluenza 3 and adenovirus reagents allowed for rapid presumptive diagnosis but were not sufficiently sensitive to replace culture.

Shell vial assays using HEp-2 and A549 cells offered no diagnostic advantage over immunofluorescent assay or conventional tube cell culture for the identification of respiratory viruses.

Immunofluorescent assay using commercial monoclonal antibodies was a sensitive and specific method for rapid diagnosis of respiratory virus infections and has the potential to replace expensive and laborious conventional culture methods.

INTRODUCTION

Etiology of Respiratory Illness

Respiratory infections are a major cause of morbidity in humans; more than 25% of all visits to or by physicians are motivated by symptoms of respiratory illness (1). Viruses, bacteria, rickettsiae, and fungi cause respiratory infections and diseases. The etiology of respiratory disease can vary from season to season, from year to year, and especially from one age group of patients to another (2). Infectious particles may be implanted directly on nasal surfaces from contaminated hands, from large respiratory droplets or from finely dispersed, aerosolized respiratory secretions which may reach the lower respiratory passages (2). Since humans continually breathe the environmental air about 20 times a minute and are in direct or indirect daily contact, it is no wonder that exposure to and infection with respiratory agents is common.

Of all microbial agents, respiratory viruses are probably the most common cause of symptomatic and possible asymptomatic infections. It is estimated that the common cold accounts for more than half of all absences from work and one quarter of the total time lost by industrial employees in the United States (1). In children and young adults, over 90% of respiratory infections are viral (3,4). Older children and adults are more likely to have less severe symptoms but are an important source of viruses for infants and the elderly (2). In infants under 2

years of age, respiratory syncytial virus and parainfluenza virus types 1 and 3 are the most important viral respiratory pathogens. Influenza is of morbidity importance in all age groups, but the mortality is highest with infections in infancy and in the aged. As advances in antiviral chemotherapy are made, concurrent advances in rapid diagnosis are needed. Current antiviral agents are virus specific and patients will benefit most by earlier diagnosis and treatment. Specific and rapid laboratory diagnostic procedures are needed for the optimal management of respiratory diseases.

Viral respiratory infections may be inapparent or symptomatic ranging from mild, self limiting diseases such as the common cold to severe and even fatal pneumonias (1). Viral respiratory disease is more common in the very young and elderly populations than in other age groups (2). These infections can be broadly categorized into several viral respiratory syndromes including diseases of the upper respiratory tract; the common cold, acute febrile pharyngitis, pharyngoconjunctival fever, acute respiratory disease, herpangina, and diseases of the lower respiratory tract including bronchitis, bronchiolitis and pneumonia, and an influenza-like syndrome. Viral infections that are confined to the respiratory tract can be caused by members of a least 5 major virus groups: Orthomyxoviridiae (influenza viruses), Paramyxoviridae (parainfluenza viruses and respiratory syncytial virus), Picornaviridae (enteroviruses and rhinovirus), Coronaviridae (coronavirus) and Adenoviridae (adenovirus) (1).

Most respiratory viruses produce illness through the direct consequences of local multiplication. Necrosis and lysis occur with

desquamation of the respiratory epithelium (2). Constitutional symptoms result from breakdown products of dying cells that are absorbed into the bloodstream. Fever is produced by the liberation of endogenous pyrogens such as interleukin 1 resulting from the anti-viral action of macrophages. Myalgia, which may be severe in respiratory infections such as influenza, results from interleukin 1-induced release of prostaglandin E2 and enhanced catabolism in muscle cells (5). The more desirable results of viral induced immune mechanisms are eradication of infection and immunity.

Usually the maintenance of low levels of secretory antibody or of cell-mediated immune sensitization provides a protective barrier to virus infection in the respiratory tract (6). The development of T cells with cytotoxic activity specific for virus-infected cells appears to be a critical factor in restricting virus infection to the respiratory tract and eradicating the infection (6). Resistance to local reinfection has generally been attributed to the presence of sufficient antibody in serum or in respiratory secretions. From the nasopharynx to the bronchiolar level, the principal antibody isotype appears to be IgA and in the lower respiratory tract, IgG antibody becomes progressively more predominant (6).

Virus-specific immune mechanisms may contribute directly to the pathogenesis of a virus infection. If humoral antibody is present in the absence of local antibody, then a more severe reaction may occur (2,6). The mechanism for this is not clear but the phenomenon has been observed in infants with actively and passively acquired respiratory syncytial virus (RSV) immunity who subsequently develop a reinfection

with this virus. One possibility is that IgG4 and IgE, as homocytotrophic antibodies, may exacerbate the symptoms of respiratory infection (6). There is evidence that the cytotoxic T-cell response to influenza virus infection may also contribute to the pathogenesis of disease. Immunocompetent mice had enhanced pulmonary pathology and shorter survival than nude mice following infection with influenza virus (7). Cell-mediated immune hyperresponsiveness has been implicated as a risk factor in the development of lower respiratory disease with RSV infection. Recipients of an inactivated RSV vaccine developed exaggerated cell-mediated immune responses following vaccination and experienced more severe forms of illness with natural-acquired infection than an unvaccinated control group (8).

The influenza viruses are enveloped, negative-strand RNA viruses containing a genome of 8 linear segments (9). Two virus-coded glycoprotein antigens are found in the surface envelope; hemagglutinin (HA) and neuraminidase (NA) (10). Cellular infection is initiated by the attachment of the hemagglutinin molecules to host cell membrane glycoproteins containing sialic acid. The hemagglutinin is a trimeric molecule that projects from the viral membrane and is composed of a fibrous stem region that supports three globular domains (11). The receptor binding domain of the hemagglutinin is a pocket of highly conserved amino acids located near the top of each of the three globular domains. Neuraminidase facilitates release of newly formed virus particles from infected cells. The surface glycoproteins of newly synthesized virions contain N-acetylneuraminic acid (NANA) as part of their carbohydrate structure. A major function of NA is the enzymatic

removal of the NANA residues to disrupt or prevent the occurrence of aggregates and thereby increase the number of free infectious particles. Both surface proteins are antigenic and antibodies to them are involved in development of acquired immunity (10). The nucleoprotein and matrix antigens of the influenza viruses are antigenically type-specific and stable and enable immune typing of the viruses into types A, B, and C (12).

Point mutations and gene reassortment among viruses of the same type, which occur in the gene segments coding for HA and NA proteins, accounts for antigenic drift and shifts (9). Such antigenic variation, particularly among type A viruses, results in the periodic epidemics and pandemics of influenza (13). Influenza infections have marked seasonality. In temperate climates, epidemics occur almost exclusively in winter months (3). Infection with influenza virus may be asymptomatic, may cause only a slight fever, or may result in the prostrating illness that occurs during epidemics of influenza (3,13). In a lengthy, prospective study of respiratory virus infections in infants and young children in a Washington, D.C. pediatric practice, influenza viruses ranked 4th in importance after RSV, the parainfluenza viruses and the adenovirus group as a cause of respiratory disease severe enough to require medical attention (13). In a 6 year study of acute respiratory illness in a small Michigan community, the influenza virus group accounted for 11.9% of all viruses isolated and was the third most frequent cause of illness (3). The major site of infection for influenza viruses is the ciliated columnar epithelial cell (14,15). Nasal and tracheal ciliated cells are extensively necrotized as early as

the first day after the onset of symptoms. Local inflammation follows and repair of the epithelium begins between the third and fifth day. After 15 days, cilia and mucus production reappear (14). Recovery from uncomplicated influenza begins 3 to 4 days after onset, although complaints of weakness, fatigue, and cough may persist for 1 week or longer. Pneumonia of viral or secondary bacterial origin is an important complication particularly in the elderly or in patients of any age with chronic debilitating illness (1). Amantadine hydrochloride provides prophylactic benefit in adults and children, however, this antiviral agent lacks activity against influenza B virus. Ribavirin has been used successfully in the treatment of both influenza A and B in adult populations (16). Yearly vaccinations of high risk groups with a continually updated, trivalent, inactivated vaccine is the primary prophylactic measure for influenza disease.

The genome of the parainfluenza viruses consists of a single-stranded, negative polarity RNA in unsegmented, linear form (17). Two surface glycoprotein spikes project from a viral envelope: a hemagglutinin-neuraminidase (HN) protein complex and a fusion (F) protein (17). The HN protein is the reactive site which binds to the cell receptors and the F protein mediates the infectivity of the virus (17). Type specific antigens in the nucleocapsid allow for differentiation into 4 distinct serotypes (18). Unlike the influenza viruses, the parainfluenza viruses are genetically stable.

The parainfluenza viruses are ubiquitous agents and among the more important viral respiratory pathogens of humans. They cause upper respiratory tract infections at all ages and are frequent causes of

acute lower respiratory tract disease in infants and young children (3,19). In an 11 year study of croup in a pediatric practice, the parainfluenza viruses constituted 74% of the agents isolated (20). The major isolate was parainfluenza virus type 1. Type 2 virus produced similar illness to type 1 but with much less frequency. In a 6 year study of parainfluenza virus type 3 infections in a Houston pediatrics practice, type 3 closely mimicked RSV in clinical manifestations in infants less than 1 year of age and was second only to RSV as a cause of pneumonia and bronchiolitis in infants (21). Type 3 infections occurred in epidemics with activity peaking in the spring. Virtually all children in the study were infected with parainfluenza virus type 3 by age 3. Parainfluenza viruses caused 16.9% of all respiratory illnesses and were second only to rhinoviruses as a cause of respiratory illness in a 6 year study of acute respiratory illness in Tecumseh, Michigan (3). Generally, most primary parainfluenza virus infections are symptomatic with a high proportion affecting the lower respiratory tract (21). Of the primary infections that involve the upper respiratory tract, most are accompanied by fever and laryngitis or tracheitis (21).

Inoculation with the parainfluenza viruses occurs via the upper respiratory tract with infection of the nasal epithelium and the nasopharynx (22). With primary infection, inflammation may be present throughout the respiratory tract in young children. Although the subglottic tissues may appear particularly involved, inflammation occurs in the conducting airways at all levels including the alveoli (22). Necrosis of the respiratory epithelium is a prominent feature in the infection process (22).

Respiratory syncytial virus, a Paramyxovirus, has envelope glycoproteins but lacks hemagglutinin and neuraminidase activity (23). Two smaller glycoproteins (F1 and F2), similar to the fusion protein of parainfluenza viruses, relate to infectivity of the virus (17,23). Group specific antigens are present in both the envelope and nucleocapsid (24).

Respiratory syncytial virus is the most important pathogen in infancy and early childhood and is the only viral agent that produces its most severe disease in the first several months of life when specific maternal antibody is uniformly present in the infant's serum (24,25,26). This viral respiratory pathogen causes sizable outbreaks of infection each year and circulates so efficiently that virtually all children in their first year of life are infected (24,25,27). Infections in children involve principally the lower respiratory tract. In a study of 13 consecutive respiratory seasons in Washington, D.C., each RSV epidemic lasted approximately 5 months with a peak incidence closely associated with a dramatic increase in the number of infants and young children who were hospitalized with lower respiratory tract disease, especially bronchiolitis and pneumonia (27). Infection with RSV occurred in 43% of inpatients with bronchiolitis, 25% of patients with pneumonia and 23% of all inpatients with acute respiratory tract disease. Respiratory syncytial virus caused 5.9% of acute respiratory illness in a small American community (3). Respiratory syncytial virus does not respect age, continuing to cause symptomatic infections throughout life (28). Viral infection in older children and adults may be the source for continued spread of RSV infection in a community (28).

Inoculation of RSV is through the upper respiratory tract with infection occurring in the respiratory epithelium (29). From the upper respiratory tract the virus may spread to the lower respiratory tract to involve the conducting airways at all levels (30). The main lesion in acute bronchiolitis is epithelial necrosis with a dense plug forming in the bronchiolar lumen leading to trapping and other mechanical interference with ventilation. These pathologic changes adversely affect the mechanics of the infant's respiration by causing a markedly increased lung volume and higher expiratory resistance. In RSV pneumonia, the characteristic finding is an interstitial infiltration of mononuclear cells (31). The lung parenchyma are edematous with areas of necrosis, leading to alveolar filling, consolidation, and collapse. The severity of symptoms following RSV infection is not due to a deficient cellular response, but rather to cell-mediated hyperresponsiveness (6).

Adenoviruses are non-enveloped, double-stranded DNA-containing viruses (32). The capsid contains 252 capsomeres and has icosahedral symmetry. The capsomeres consist of 240 hexons and 12 pentons with a projecting fiber on each penton. Hexons are group-specific antigens and induce neutralizing antibodies; the fibers are largely responsible for type-specific antibody induction. The pentons have mixed functions and are especially active in hemagglutination (33). A monoclonal antibody has been produced and characterized that reacts with a common hexon epitope of all 41 adenovirus serotypes (34). Adenoviral infections accounted for 2 to 5% of total respiratory illness in a study in Seattle, Washington (35), and adenoviruses were isolated from 4.5% of

patients exhibiting acute respiratory illness in a Tecumseh, Michigan study (3). In a 10 year study of pediatric respiratory disease in Washington, D.C., adenoviruses accounted for approximately 7% of these diseases with a clinical distribution in these percentages: 12.4% of mild upper respiratory tract infection, 10.4% of pharyngitis, 6.3% of croup, 10.6% of bronchiolitis and 11.6% of pneumonia (26).

In most instances, initial adenovirus infection occurs in the respiratory tract and involves the mucus membranes of the nose, oropharynx and conjunctiva (36). In tracheal organ cultures, the growth of adenovirus 7 was characterized by an initial focal cytopathology that quickly progressed to involve the whole epithelium (37). Frequently the cilia of inclusion-bearing cells were found to be intact.

Picornaviruses are small, nonenveloped viruses with a genome consisting of a linear, single-stranded, positive-sense RNA molecule (38). The family Picornaviridae includes 2 genera of human pathogens: rhinoviruses and enteroviruses. The enteroviruses (polioviruses, echoviruses, coxsackie A and B viruses, enterovirus serotypes 68,69,70, and 71, and hepatitis A virus) are found mainly in the gastrointestinal tract and are stable at pH 3.0; the rhinoviruses are found primarily in the nose and are inactivated at pH 3.0 (38). The protein coat of the picornaviruses forms an icosahedral shell consisting of sixty protomers arranged in twelve pentameric units. Each protomer contains one copy of each of the viral proteins VP1, VP2, VP3, and VP4. In both poliovirus and rhinovirus capsids there is a deep depression surrounding the vertex of each of the pentameric units. These depressions have been proposed to be sites that bind to the host receptor. The receptor site on the

host cell for rhinovirus and poliovirus is a member of the immunoglobulin superfamily (11).

Although more than 100 serotypes constitute the genus Rhinovirus, no common antigen is found in all serotypes (39). Rhinoviruses are associated primarily with mild upper respiratory tract disease (the common cold) and are believed to cause 30 to 50% of all acute respiratory illness (40,41).

Rhinovirus infection occurs via the respiratory route and, despite the marked local response to infection, little destruction of the nasal mucosal epithelium is noted (42). Only a few rhinovirus-containing ciliated epithelial cells were shed from the nasal membranes of laboratory-infected volunteers (43). In this study, the acute stage of rhinitis was characterized by edema of the mucus membrane with exudation of serous and mucinous fluid, i.e. the common cold.

Although there are some minor cross reactions between several coxsackievirus and echovirus groups, there are no common group antigens of diagnostic importance (44). Coxsackievirus A21 is the only enterovirus that clearly qualifies as a common cold virus, producing epidemics of mild respiratory illness in military populations (45). Pharyngitis is a common clinical manifestation of coxsackie and echoviral infections (45). Probably all enteroviruses on occasion cause mild pharyngitis which is usually abrupt in onset, without prodrome and accompanied by fever (45). Croup, bronchitis, bronchiolitis and pneumonia have been sporadically associated with coxsackievirus and echoviruses (45). Epidemic myalgia (pleurodynia or Bornholm disease) is characterized by a sudden onset of fever and chest pain which likely

results from viral infection of muscle cells (46). The major etiologic agents in epidemic myalgia are coxsackieviruses B3 and B5 (46). Herpangina, with symptoms of fever, pharyngitis, dysphagia, abdominal pain, and vomiting is caused by coxsackie A viruses in young children (45). Enteroviruses contribute significantly to the burden of disease in a community; 4.3% of the respiratory illnesses observed during a 6 year study in Tecumseh, Michigan were caused by enteroviruses (3).

Transmission of enteroviruses is from person to person by fecal-oral and possibly oral-oral routes (45). Following initial acquisition of virus, implantation occurs in the pharynx and the lower alimentary tract (45). The pathologic findings in patients with mild respiratory tract infections have not been described.

Human coronaviruses are enveloped, single-stranded, positive-sense RNA viruses with club-shaped surface projections that give the appearance of a solar corona (47). Because of the fastidious growth requirements of coronaviruses, successful isolation of most serotypes is dependent upon the utilization of organ cultures of human ciliated respiratory epithelial tissues (48). Coronaviruses are the second most important viral group responsible for the common cold and infection is clinically manifested by a general malaise, headache, rhinorrhea, and a sore throat (49).

Coronavirus infection begins in the upper respiratory tract, destroying the ciliated epithelium of the trachea and nasal mucosa (50). Infections are characterized by sharp midwinter outbreaks between December and April (49).

The rapid diagnosis of RSV, influenza A and B virus, parainfluenza

virus types 1, 2, and 3, and adenovirus infections has received a great deal of attention because of the severe disease associated with these agents. Fortunately, these viruses exhibit group specific antigens and rapid immunologic identification can be made. The impetus to develop rapid diagnostic techniques for the enteroviruses, rhinoviruses and coronaviruses has been blunted by the fact that these viruses lack group specific antigens. Enteroviruses, rhinoviruses and coronaviruses are generally of little concern in the laboratory because of an absence of immunologic methods for group specific identification, fastidious culture requirements, and a general association with mild upper respiratory tract symptoms.

Diagnosis of Respiratory Virus Infections

Serology and isolation in cell culture are the traditional methods for the laboratory diagnosis of viral respiratory illnesses. Although these time-honored tests remain the "gold standard" against which new methodologies are compared, they are inherently slow; acute- and convalescent-phase sera must be separated by at least 7 days, and the median time for detection of all viruses in cell culture is 4 days (51). With the advent of specific antiviral therapy, more rapid methods of viral diagnosis are highly desirable.

Circulating antiviral antibodies, particularly neutralizing antibodies, are widely accepted as evidence of recent or past infection with a particular virus. Demonstration of a fourfold increase in specific antiviral antibody is considered diagnostic of a recent

infection. Serology has the advantage of establishing a diagnosis when virus isolation either is negative or cannot be attempted. A negative viral serologic test can often exclude a specific virus from etiologic possibilities, whereas failure to isolate the virus or to detect specific viral antigens in patients specimens cannot. On the other hand, all serologic diagnoses must be considered presumptive to some degree because serologic cross reactions within many virus groups occur. The delay in diagnosis obviates conventional serologic methods as useful for guiding decisions concerning prompt initiation of specific antiviral chemotherapy.

Methods that identify viruses in cell culture before observation of cytopathic effect have been studied as a way to decrease time for detection of viruses. Hemadsorption, which detects influenza A, influenza B and parainfluenza type 1, 2, and 3 viral hemagglutinin in primary monkey kidney cells, showed that 38% of all viruses could be detected by 24 hours, 69% could be detected by 48 hours and 96% could be detected by 5 days (52). The poorest recovery was for parainfluenza type 2 virus; 82% detection at 5 days. All viruses were detected by day 10.

Immunologic techniques such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence microscopy (IFM) have been used to detect respiratory virus antigens in clinical specimens with results available in a matter of hours.

Most ELISAs that detect respiratory virus antigens employ a capture antibody bound to a solid phase (53). Incubation with a clinical specimen results in binding of viral antigen to the bound antibody. The

captured viral antigen is then detected by another specific antibody which is conjugated to an enzyme. The bound enzyme is detected and quantitated by the development of color when an appropriate substrate is added. These assays have objective endpoints and can be performed with automated instrumentation, making them ideal for testing large numbers of samples. Assays have been constructed using monoclonal or polyclonal antibodies.

Immunofluorescence microscopy (IFM) is the most commonly practiced rapid technique in diagnostic laboratories today (54). The introduction of monoclonal antibodies (MAB) has improved the reliability and broadened the acceptance of IFM as a rapid viral diagnostic tool. Many of the MABs used in IFM are commercially available, permitting the wide dissemination of standardized reagents that have greater specificity and much lower background fluorescence than polyclonal sera (54). Because the endpoint is subjectively determined, diagnosis by IFM requires experience and careful interpretation.

Although rapid, the usefulness of immunologic tests in clinical practice must be determined. A predictive value model of diagnosis describes the explicit relationship between test results and the state of health (55). A true negative (TN) result occurs when a test is negative and the patient's state of health is normal. Conversely, when the test is positive and the patient has disease, the patient is correctly identified as having the disease. This constitutes a true positive (TP) result. Since uncertainty accompanies even the best clinical tests, false positive (FP) and false negative (FN) test results can occur. Patients without disease but demonstrating a positive test

result are categorized as being FP and patients with disease and demonstrating a negative test are categorized as FN. The predictive value model can be used in diagnostic medicine to evaluate whether a positive or a negative result is correct (Table 1). The predictive value of a positive test result is the percentage of TP results with respect to all individuals with positive test results. Similarly, the predictive value of a negative test result is the percentage of TN test results in all individuals who have negative test results.

Two additional parameters, sensitivity and specificity can be calculated (Table 1) and are assumed intrinsic properties of the test. A test's sensitivity is the percentage of known diseased subjects with positive test results among all the diseased subjects tested. The specificity of a test is the percentage of subjects with negative results among all known nondiseased subjects tested.

An important factor affecting the usefulness of a test result is prevalence which is defined as the percentage of the population that exhibits the disease at a point in time divided by the total (both diseased and nondiseased) population. The predictive value model can be expressed algebraically as a function of disease prevalence and the sensitivity and specificity of a diagnostic test (Table 2) (55). As the prevalence of a disease increases in the tested population, the predictive value of a positive test result increases. Conversely, as the prevalence of disease in the test population increases, the predictive value of a negative test result decreases because of the proportionately greater number of false negative test results.

A fluorescent focus chamber slide assay utilizing murine monoclonal

Table 1. Predictive value model showing the relationship between test results and state of health (55).

Group	Category of Positive Result	Category of Negative Result
Diseased (Culture Positive)	TP	FN
Nondiseased (Culture Negative)	FP	TN

TP - True Positive
FN - False Negative
FP - False Positive
TN - True Negative
Sensitivity = TP/(TP + FN)
Specificity = TN/(TN + FP)
Predictive Value Positive = TP/(TP + FP)
Predictive Value Negative = TN/(TN + FN)

Table 2. Predictive value model expressed algebraically (55).

Group	Positive Result	Negative Result
Diseased	(prev)(sens)	(prev)(1 - sens)
Nondiseased	(1 - prev)(1 - spec)	(1 - prev)(spec)

Prevalence (prev) = (TP + FN)/(TP + FN + TN + FP)
Sensitivity (sens) = TP/(TP + FN)
Specificity (spec) = TN/(TN + FP)
Predictive Value Positive = [(prev)(sens)]/[(prev)(sens) + (1 - prev)(1 - spec)]
Predictive Value Negative = [(1 - prev)(spec)]/[(prev)(1 - sens)] + [(1 - prev)(spec)]

antibodies directed against conserved epitopes of influenza A and B viruses was compared to virus isolation using Madin-Darby canine kidney cells (MDCK) (56). Clinical specimens were inoculated onto MDCK monolayers in chamber slides, incubated for 24 hours and stained with monoclonal antibody. Compared with immunofluorescent staining of conventional cell culture monolayers, 24 hour cell culture chamber slides had a sensitivity of 75% and a specificity of 95%. A retrospective study evaluating the efficacy of influenza type A- and B-specific monoclonal antibodies in an indirect immunoperoxidase (IPA) staining procedure was performed on primary monkey kidney cell monolayers 24 hours postinoculation with stored clinical specimens (57). The IPA stain detected influenza virus is 86% of stored clinical specimens from which the virus was reisolated. An enzyme immunoassay (EIA) employing the same monoclonal antibodies as described in the IPA procedure was used to detect influenza viruses reisolated from clinical specimens in tissue culture (45). At 48 hours postinoculation, 64% of H1N1 and 94% of H3N2 influenza A specimens and 79% of influenza B specimens were detected.

Enzyme-linked immunosorbent assay (ELISA) is a highly specific method of determining the presence of RSV antigen is nasopharyngeal secretions (58-63). Sensitivities of this assay when compared to cell culture have ranged from 61 to 94%. Although immunofluorescence appears to be slightly more sensitive than ELISA for the detection of RSV in clinical specimens, ELISAs require no special equipment and only minimal training (63).

Detection of influenza A virus in nasal wash specimens by one ELISA

was not sufficiently sensitive nor specific for routine laboratory diagnosis of influenza (64). Only 10 of 20 culturally-proven infections were positive in the test and 2 of 13 patients with rhinovirus infections were falsely positive. Using another ELISA procedure, Harmon and Pawlik were able to detect influenza A and adenovirus antigens in nasal wash and throat swab specimens with only a 62% and 53% efficiency, respectively (65). Neither of these ELISA tests are commercially available.

August and Warford evaluated a commercial ELISA test for the detection of adenovirus in conjunctival, respiratory and fecal specimens (66). Of 45 culture positive specimens, 25 (56.8%) were positive by ELISA. Wiley and colleagues demonstrated an overall sensitivity and specificity of 69% and 100%, respectively, for detection of adenovirus from ocular specimens (67). Despite the low sensitivity of this ELISA test, advantages of the test include the ability to detect the noncultivable adenovirus 40 and 41, and the potential for automation.

Centrifugation of clinical specimens onto monolayers in shell vials (shell vial assay) is thought to enhance the infectivity of viruses. Rapid techniques for the detection of cytomegalovirus and herpes simplex virus in shell vial cultures 16 hours postinoculation using monoclonal antibodies with an indirect fluorescent antibody (IFA) procedure have yielded results superior to those obtained with conventional tube cell cultures (68,69).

Several studies applying the shell vial assay for the detection of respiratory viruses have been undertaken. Epsy and colleagues detected only 56% of influenza strains by IFA in the shell viral assay (SVA) 24

hours postinoculation as compared to conventional primary monkey kidney tube cell cultures (70). At 48 hours postinoculation, the sensitivity increased slightly to 60%. The SVA was never positive in the absence of the isolation of influenza virus in conventional tube cell cultures. Using the same antibodies, Stokes and colleagues demonstrated an 84% sensitivity and 100% specificity with a MDCK-seeded SVA. A monoclonal antibody specific for the adenovirus group-reactive hexon antigen was used for the detection of adenoviruses by IFA 24 and 48 hours postinoculation of HEp-2 monolayers in shell vials (71). Of 31 adenovirus isolates, 16 (52%) and 30 (97%) were detected after incubation for 24 hours and 48 hours, respectively. August and Warford, using a commercial monoclonal antibody with a 72 hour SVA, were able to demonstrate an 86% sensitivity and 100% specificity compared to human foreskin fibroblast tube cell cultures (66).

Efficient immunologic detection of respiratory virus antigen in direct clinical specimens is dependent upon 2 factors: the availability of reliable antisera and collection procedures that result in specimens containing detectable amounts of viral antigen.

Suitable monoclonal antibodies would be an obvious advantage over polyclonal sera for laboratory diagnosis. They would provide long-term reagent consistency and could be used without adsorption to remove cross reacting tissue-specific antibodies. In a study comparing polyclonal antiserum to monoclonal antibodies for the rapid detection of influenza A virus in respiratory specimens by immunofluorescence, monoclonal antibodies were more sensitive and accurate than polyclonal antiserum, and the monoclonal preparation was easier to interpret (72).

Infection with respiratory viruses occurs via the respiratory epithelium and viral antigen can be demonstrated in sloughed columnar epithelium (14,30,37,43). Nasal wash specimens are superior to either throat swabs or nasopharyngeal swabs for RSV isolation (73). An aspiration method utilizing a portable suction catheter and secretion trap for collection of nasopharyngeal secretions was used successfully to collect specimens for direct antigen detection and culture (74). An effective and simple method for collection of nasal wash specimens that does not require a suction apparatus was described by Hall (75). Specimens obtained by this method contained titers of RSV that were on average 500-fold higher than those obtained by using a conventional nasopharyngeal swab method. Alternatively, nasopharyngeal swab specimens compared favorably with nasopharyngeal suction-collected specimens with respect to the number of specimens suitable for immunofluorescence (76). For the detection of virus antigens in direct clinical specimens by immunofluorescence, satisfactory specimens should contain at least 1 ciliated epithelial cell per 400X field (76,77).

The use of immunofluorescence for the rapid detection of respiratory viruses in cells shed from the nasopharynx during infection is a sensitive diagnostic procedure (77). A more widespread use of immunofluorescence has been limited by a shortage of antisera of acceptable quality (78). Table 3 summarizes the efficiency of immunofluorescence for detection of respiratory viruses from clinical specimens. These data suggest the immunofluorescence could be used as an alternative to culture for RSV (63). The specificity for the remaining viruses appears sufficiently great to allow a confident

Table 3. Efficiency of immunofluorescence for detection of respiratory viruses in clinical specimens.

Virus	Antibody Type/Source	Method	Sensitivity	Specificity	Reference
Influenza A	P(1)/C(2)	IFA(3)	43	100	79
	P/C	DFA(4)	88	88	76
	P/C	DFA	73	90	74
	M(5)/PR(6)	IFA	38	91	56
	P/C	IFA	46	75	72
	M/PR	IFA	69	86	72
Parainfluenza					
Type 1	P/C	DFA	71	99	76
	P/C	IFA	63	98	79
Type 2	P/C	DFA	76	100	76
Type 3	P/C	DFA	69	99	76
	P/C	IFA	31	100	79
Adenovirus	P/C	DFA	100*	99	76
	P/C	IFA	28	100	79
	P/PR	IFA	62	-(7)	80
Respiratory syncytial	P/C	DFA	92	95	76
	P/C	IFA	93	94	79
	P/C	IFA	95	96	81
	P/C	DFA	95	91	82
	P/PR	IFA	92	94	83
	P/C	DFA	94	69	84
	M/PR	IFA	95	87	85
	M/PR	IFA	97	89	86

(1) - Polyclonal
(2) - Commercial
(3) - Indirect Fluorescent Antibody
(4) - Direct Fluorescent Antibody
(5) - Monoclonal
(6) - Private
* - Low Numbers
(7) - Undetermined

presumptive diagnosis. The sensitivity for viruses other than RSV needs to be improved. It is suggested that immunofluorescence be an adjunct to, and not a replacement of, appropriate cell culture isolation systems (79).

Rapid diagnostic procedures are not available for coronaviruses, rhinoviruses and enteroviruses. Coronaviruses generally require tracheal organ culture for in vitro isolation and commercially produced antibodies are not available for detection of this virus by immunofluorescence or ELISA. Rhinoviruses and enteroviruses lack a common antigen, therefore immunofluorescence and ELISA procedures have not been developed that react broadly enough for diagnosis of all serotypes. Since respiratory infections with coronaviruses and rhinoviruses are mild and usually do not require hospitalization, they are not a major concern in the diagnostic virology laboratory (78).

Laboratory directors must consider the availability, adaptability and efficiency of a test when selecting a procedure for rapid viral diagnosis. Two methodologies were considered for this study: ELISA and immunofluorescence. While commercial IFA reagents were available for RSV, influenza A and B, parainfluenza virus types 1, 2, and 3, and adenovirus, the only commercially available ELISA reagents were for RSV and adenovirus. Immunofluorescence microscopy had been performed at the Quain and Ramstad clinic for several years, therefore, no additional equipment was needed to perform immunofluorescence for detection of respiratory virus antigens. Whereas a separate ELISA must be performed for each specific virus, a pool reagent could be used to screen for several viruses by immunofluorescence. For these reasons,

immunofluorescence was selected as the methodology for this study.

The purpose of this study was twofold: first, to determine the epidemiology of specific respiratory viruses in the Bismarck, North Dakota area, and second, to determine the efficiency and usefulness of new monoclonal antibodies and shell vial culture for detection of respiratory viruses in clinical specimens. Viral prevalence was determined by viral culture of NPW and NPS specimens and of throat swabs previously cultured for GAS. The ability of shell vial cultures and immunofluorescence to detect viruses in NPW and NPS specimens was compared to isolation of the viruses in conventional tube cell culture. It was our intent to evaluate rapid diagnostic procedures that are applicable to small hospital laboratories, and provide a rapid laboratory diagnosis for the management and treatment of viral respiratory diseases.

MATERIALS AND METHODS

Cell Cultures

Primary monkey kidney (PMK) cells in 16 x 125 mm glass culture tubes were obtained from Bartels Immunodiagnostics, Incorporated (Microscan Division, Baxter Healthcare Corporation, West Sacramento, California). Cell lines utilized in the study included human laryngeal epidermoid carcinoma (HEp-2, ATCC CCL 23), human lung carcinoma (A549, ATCC CCL 185) and human embryonal diploid lung (MRC-5, ATCC CCL 171). Cell lines were grown to confluency in 75 cm3 culture flasks (Corning Glass Works, Corning, New York), washed twice in calcium- and magnesium-free phosphate buffered saline, trypsinized (0.25%) for 10 minutes at 35C and subcultivated at a ratio of 1:8, 1:8, and 1:5, respectively. The HEp-2 and A549 cell lines were also subcultured to 1 dram shell vials containing circular 12 mm glass coverslips. Cells were grown in Hank's minimal essential medium (H-MEM, Gibco Laboratories, Grand Island, New York) supplemented with 10% newborn calf serum (NCS, Irvine Scientific, Santa Ana, California) and the cells were maintained in Earle's minimal essential medium (E-MEM, Gibco Laboratories, Grand Island, New York) supplemented with 2% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, New York). Culture refeeding medium consisted of E-MEM supplemented with 3% antibiotic solution containing penicillin (10,000 Units/ml), streptomycin (10,000 Units/ml) and amphotericin B (25 ug/ml).

The Study Population and Specimen Procurement

From December, 1987, through June, 1989, respiratory specimens were submitted to the Microbiology Laboratory at the Quain and Ramstad Clinic, Bismarck, North Dakota. Included in the study were nasopharyngeal swab (NPS), nasopharyngeal wash (NPW) and throat swab (TS) specimens collected by physicians and nursing personnel. Specimens were collected from clinic outpatients or were referred to the laboratory from neighboring clinics and hospitals.

Collection and Storage of Specimens

Nasopharyngeal Wash (NPW)

The method described by Hall, et al. (75) was used to collect NPW specimens. At the patient's bedside, a 1-ounce rubber ear and ulcer syringe (Davol, Incorporated, Cranston, Rhode Island) was loaded with 3 to 5 ml of sterile phosphate buffered saline (PBS). With the patient's head tipped back at about 70 degrees, the bulb was inserted until it occluded the nostril. With one complete squeeze and release, the nasal wash was collected in the bulb. The bulb contents were then emptied into a sterile screw-capped tube and transported to the laboratory.

Nasopharyngeal Swabs (NPS)

Nasopharyngeal swab specimens were collected on Mini-tip culturettes (Marion Scientific, Kansas city, Missouri) by inserting the swab 4 to 5 cm into the nostril. The swab was left in place for approximately 5 sec, carefully removed, and immediately placed into the culturette container with modified Stuart's medium for transport to the laboratory. Collection of 2 NPS specimens was strongly encouraged in order to ensure a sufficient number of ciliated epithelial cells for microscopic examination.

Throat Swabs (TS)

Throat swab specimens were collected on regular culturettes (Marion Scientific, Kansas City, Missouri) by vigorously swabbing the tonsillar crypts and pharynx. Swabs were placed into the culturette container with Stuart's medium for transport to the laboratory.

All TS, NPW and NPS specimens were stored at 2 to 8C until processed, usually within 24 hours of receipt.

Detection of Group A Streptococcus (GAS) by Immunofluorescence

Throat swab specimens were inoculated onto tryptic soy agar plates containing 5% defibrinated sheep red blood cells (DiMed Corporation, St. Paul, Minnesota), streaked for isolation, and incubated anaerobically for 16 to 24 hours in a Gas Pak jar (BBL Microbiological Systems, Becton

Dickinson and Company, Cockeysville, Maryland). Beta hemolytic colonies were suspended in a drop of PBS on a glass slide, and the slide was air dried and fixed with cold ethanol for one minute. One drop (approximately 50 ul) of lapine anti-GAS antiserum labeled with fluorescein isothiocyanate (BBL Microbiological Systems, Becton Dickinson and Company, Cockeysville, Maryland) was added to each slide. After 30 minutes incubation at room temperature, the slide was washed in PBS for 10 minutes, air dried, mounted with buffered glycerol, and read using an immunofluorescent microscope. Cocci exhibiting 4+ fluorescence were interpreted as being positive for GAS.

After being processed for GAS, TS specimens were randomly selected for viral culture. Viruses, not screened for by immunofluorescent assay but isolated in cell culture, were identified by cytopathogenic effect in cell culture. Clumping and rounding of MRC-5 and HEp-2 cells with some syncytial formation was indicative of herpes simplex virus and generalized cell rounding and destruction, primarily in PMK cells, signified the presence of an enterovirus.

Immunofluorescent Microscopy

All slides and coverslips were read at a magnification of 200X to 400X with incident light illumination using a Zeiss fluorescent microscope (Carl Zeiss, Incorporated, Thornwood, New York) equipped with a tungsten halogen lamp, a 495 nm excitation filter and a 520 nm emission filter.

Processing of Throat Swabs, Nasopharyngeal Swabs and Nasopharyngeal Washes in Preparation for Cell Culture and Immunofluorescent Staining

Nasopharyngeal washes, NPS in 2 ml PBS, and TS in 2 ml PBS were vortexed (10 seconds, highest speed setting) to create a primary cell suspension (PCS). One ml aliquots of the PCS were stored at -70C. The remaining PCS was centrifuged for 5 minutes at 1500 x g in a Beckman model TJ-6R centrifuge (Beckman Instruments, Incorporated, Palo Alto, California) to pellet the cells. The supernatant fluid was stored at 2 to 8C and used to inoculate tube cell culture. Because they contained squamous epithelial cells rather than ciliated epithelial cells, the TS cell pellets were not suitable for direct specimen testing and were stored at -70C after inoculation of tube cell culture. The remaining supernatant fluid was decanted and NPW and NPS cell pellets were resuspended in the residual PBS. Direct patient specimens (DPS) were prepared by placing 1 to 2 drops of the cell suspension onto glass slides (wettable, acetone resistant; Roboz Surgical Instrument Company, Incorporated, Washington, D. C.). The slides were then air dried and fixed in cold acetone for 10 minutes.

Inoculation and Fixation of Cell Cultures

Conventional Tube Cell Culture

The maintenance medium was decanted from tubes of PMK, HEp-2, and MRC-5 cells and each cell type was inoculated with 0.2 to 0.5 ml of

PCS supernatant fluid. After adding 2 ml of refeeding medium to each tube, the cultures were incubated at 35C and examined daily for cytopathogenic effect (CPE).

At 10 days or when CPE was evident, the cell monolayers (CM) were scraped into the culture medium with a sterile disposable pipette, vigorously pipetted to suspend the cells, and centrifuged (1500 x g, 5 min). The supernatant fluid was stored at -70C, and the cell pellet was resuspended in 2 ml PBS and centrifuged (1500 x g, 5 min). After decanting the supernatant fluid, the cell pellet was resuspended in residual PBS, and the cells were spotted to a glass slide, air dried and fixed in cold acetone for 10 minutes.

Shell Vial Cultures

Depending upon the test protocol, 0.2 ml of the PCS was inoculated either into duplicate A549-seeded shell vials or into a single HEp-2-seeded shell vial. After centrifugation (1500 x g, 30 min, 25C), the vials were gently agitated, and the excess inoculum was removed with a disposable pipette. One ml of refeeding medium was added to each vial and the cultures were incubated at 35C.

The HEp-2-seeded shell vial cultures were processed 48 hours postinoculation. One of the A549-seeded shell vial cultures was processed at 48 hours postinoculation and the second shell vial was processed at 5 days postinoculation only if virus antigen was present in the first vial. After removal of the maintenance medium, the coverslips

were washed twice in one ml volumes of PBS (5 minutes each) and fixed in 1 ml of cold acetone for 10 minutes.

Sources of Antibodies and Immunofluorescent Staining Procedures

Indirect Fluorescent Antibody (IFA) Reagents

The IFA reagents (Table 4) were commercially available from Bartels Immunodiagnostics, Incorporated (Baxter Healthcare Corporation, Microscan Division, West Sacramento, California) and were supplied as a panel (Bartels Indirect Fluorescent Antibody Viral Respiratory Panel). Components of the panel included murine monoclonal antibodies specific for respiratory syncytial virus (BIFA-RSV), influenza A (BIFA-FA), influenza B (BIFA-FB), parainfluenza virus types 1, 2, and 3 (BIFA-P1, BIFA-P2, and BIFA-P3, respectively), and adenovirus (BIFA-Ad), a murine monoclonal antibody pool and a fluorescein isothiocyanate- (FITC-) labeled rabbit anti-mouse antibody with Evan's blue counterstain (conjugate). The pool reagent contained antibodies specific for RSV, influenza A and B, parainfluenza virus types 1, 2, and 3, and adenovirus. The IFA reagents were used to stain DPS, CM, and A549 shell vial coverslips.

Indirect Fluorescent Antibody Staining of Direct Patient Specimens (DPS) and Tube Culture Monolayers (CM)

Specimens were stained first with the pool reagent for virus

Table 4. Commercial sources of monoclonal antibodies used in the study.

Method	Specificity of Antibody	Manufacturer	Designation
DFA(1)			
	Respiratory Syncytial Virus	B(2)	BDFA-RSV
	Respiratory Syncytial Virus	W(3)	WDFA-RSV
	Influenza A Virus	W	WDFA-FA
	Influenza B Virus	W	WDFA-FB
	Parainfluenza type 1 Virus	W	WDFA-P1
	Parainfluenza type 2 Virus	W	WDFA-P2
	Parainfluenza type 3 Virus	W	WDFA-P3
	Adenovirus	W	WDFA-Ad
IFA(4)			
	Respiratory Syncytial Virus	B	BIFA-RSV
	Influenza A Virus	B	BIFA-FA
	Influenza B Virus	B	BIFA-FB
	Parainfluenza type 1 Virus	B	BIFA-P1
	Parainfluenza type 2 Virus	B	BIFA-P2
	Parainfluenza type 3 Virus	B	BIFA-P3
	Adenovirus	B	BIFA-Ad

(1) - Direct Fluorescent Antibody
(2) - Bartels Immunodiagnostics
(3) - Whittaker M. A. Bioproducts
(4) - Indirect Fluorescent Antibody

antigen. If positive, the specimens were stained with the individual viral reagents for specific identification. One drop (approximately 50 ul) of pool or virus specific reagent was added to each well. The slides were incubated for 30 minutes at 35C in a humidified chamber, washed in PBS with gentle agitation for 10 minutes and air dried. One drop (approximately 50 ul) of conjugate was added to each well and the slide was incubated for 30 minutes at 35C in a humidified chamber. After washing with agitation in PBS for 10 minutes, the slide was air dried and mounted with buffered glycerol.

Indirect Fluorescent Antibody Staining of Shell Vial Coverslips

Coverslips were stained for virus antigen using the pool reagent. If positive, the cells on the coverslip of the second shell vial were harvested, transferred to slides and stained with virus-specific IFA reagents as described above under the section: IFA staining of DPS. All staining and washing procedures were done in the shell vials. Two drops (approximately 100 ul) of pool reagent was added to each shell vial and the recapped vials were incubated at 35C for 30 minutes. The pool reagent was removed and the coverslips were washed twice in 1 ml of PBS (5 minutes each). A bent-tip needle and fine-tip forceps facilitated removal of the coverslips from the shell vials. The coverslips were air dried (cell side up) and mounted (cell side down) with phosphate buffered glycerol.

Direct Fluorescent Antibody (DFA) Reagents

The DFA reagents (Table 4) were commercially available from Bartels Immunodiagnostics, Incorporated (Baxter Healthcare Corporation, Microscan Division, West Sacramento, California) and from Whittaker (M. A. Bioproducts, Walkersville, Maryland). All reagents were FITC-conjugated monoclonal antibodies and included: Bartels and Whittaker anti-RSV (BDFA-RSV and WDFA-RSV, respectively), Whittaker anti-influenza A (WDFA-FA), anti-influenza B (WDFA-FB), anti-parainfluenza types 1, 2, and 3 (WDFA-P1, WDFA-P2, and WDFA-P3, respectively), and anti-adenovirus (WDFA-Ad). The BDFA-RSV reagent was used to stain HEp-2-seeded shell vial cultures and all DFA reagents were used for staining of DPS.

Direct Fluorescent Antibody Staining of Direct Patient Specimens (DPS)

One drop (approximately 50 ul) of each DFA reagent was added to specific wells of the DPS slides. After incubation at 35C for 30 minutes in a humidified chamber, the slides were washed with gently agitation in PBS for 10 minutes, air dried and mounted with phosphate buffered glycerol.

Direct Fluorescent Antibody Staining of HEp-2-Seeded Shell Vial Coverslips

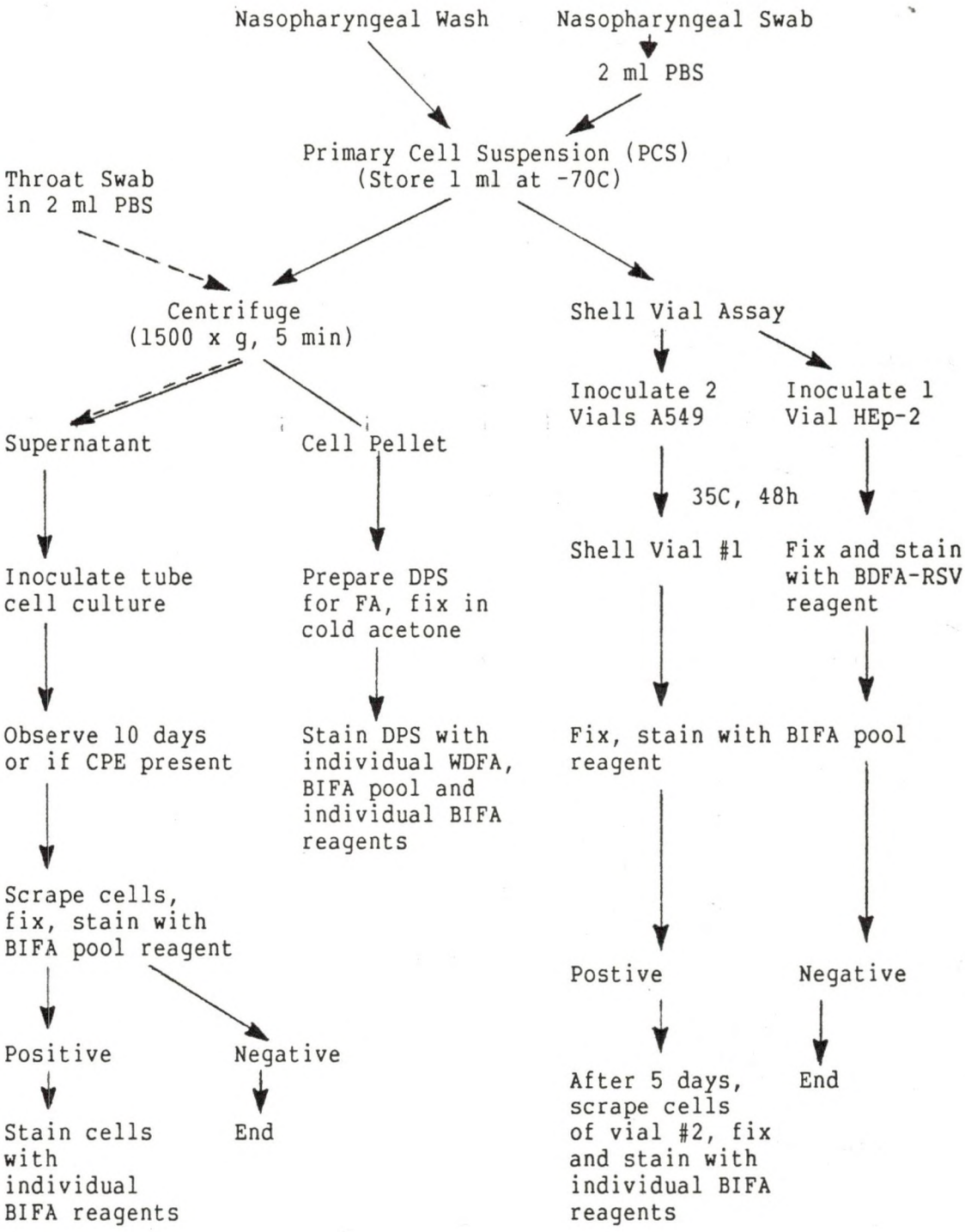
Staining and washing of the coverslips were performed directly in the shell vials. Two drops (approximately 100 ul) of the BDFA-RSV reagent was added to each shell vial and the recapped vials were incubated for 30 minutes at 35C. The reagent was removed and the coverslip was washed twice in 1 ml volumes of PBS (5 minutes each). The coverslips were removed and mounted as described above under the section: IFA staining of shell vial coverslips.

A flow diagram summarizing the protocols used in processing TS, NPS and NPW specimens is given in Figure 1.

Interpretation of Immunofluorescent Stains

A positive immunofluorescent result (virus antigen present) was dependent upon observation of specific intracellular fluorescence patterns: cytoplasmic for RSV and parainfluenza viruses; nuclear for influenza viruses; and cytoplasmic and nuclear for adenovirus. At least 2 fluorescent cells per well were required for a positive result when reading DPS, CM, and shell vial coverslips.

Figure 1. Flow diagram of processing protocols for throat swab, nasopharyngeal wash and nasopharyngeal swab specimens.


Nasopharyngeal Wash
Nasopharyngeal Swab
2 ml PBS
Primary Cell Suspension (PCS)
(Store 1 ml at -70C)
Throat Swab
in 2 ml PBS
Centrifuge
(1500 x g, 5 min)
Shell Vial Assay
Inoculate 2
Vials A549
Inoculate 1
Vial HEp-2
Supernatant
Cell Pellet
35C, 48h
Shell Vial #1
Fix and stain
with BDFA-RSV
reagent
Inoculate tube
cell culture
Prepare DPS
for FA, fix in
cold acetone
Observe 10 days
or if CPE present
Stain DPS with
individual WDFA,
BIFA pool and
individual BIFA
reagents
Fix, stain with BIFA pool
reagent
Scrape cells,
fix, stain with
BIFA pool reagent
Postive
Negative
Positive
Negative
End
Stain cells
with
individual
BIFA reagents
After 5 days,
scrape cells
of vial #2, fix
and stain with
individual BIFA
reagents

Data Analysis

The relative efficiencies of immunofluorescent reagents were determined by using tube cell culture as the standard. Sample pairs which were both culture-positive and immunofluorescence-positive were considered to be true positives (TP); culture-negative and immunofluorescence-positive pairs were termed false-positives (FP); culture-positive and immunofluorescence-negative pairs were termed false-negative (FN) and specimen pairs that were culture-negative and immunofluorescence-negative were termed true negative (TN). Sensitivity (sens), specificity (spec), and predictive values were calculated as follows: sensitivity = (TP/TP + FN) x 100, sensitivity = (TN/TN + FP) x 100, positive predictive value = (TP/TP + FP) x 100, and negative predictive value = (TN/TN + FN) x 100. Predictive values as a function of disease prevalence (prev) were calculated as follows: positive predictive value = [(prev)(sens)]/[(prev)(sens) + (1 - prev)(1 - spec)] and predictive value negative = [(1 - prev)(spec)]/[(prev)(1 - sens)] + [(1 - prev)(spec)].

RESULTS

The Study Population

The period of study was from November, 1987, through June, 1989. Of a total of 1007 respiratory specimens included in the study, 480 consisted of throat swabs (TS) specimens, 237 were nasopharyngeal swabs (NPS), and 290 were nasopharyngeal washes (NPW). The age and sex distribution of the patients from whom NPW/NPS and TS specimens were collected is defined in Figures 2 and 3, respectively. Of all patients, 48% were male and 52% female. Native American patients comprised 4.6% of the population. Over half (55.8%) of the nasopharyngeal specimens were obtained from hospitalized patients. The majority of the nasopharyngeal specimens were collected from patients less than or equal to 6 years of age. The majority of the TS specimens were obtained from patients between the ages of 1 and 49.

Epidemiology of Group A Streptococcus and Respiratory Viruses in Bismarck, North Dakota

Isolation of Group A Streptococcus (GAS) and Viruses from Throat Swabs

Throat swabs which were submitted for identification of GAS

Figure 2. Age and sex distribution of patients from whom nasopharyngeal wash and nasopharygeal swab specimens were obtained. Symbols: Males, (///); Females, (\\\).

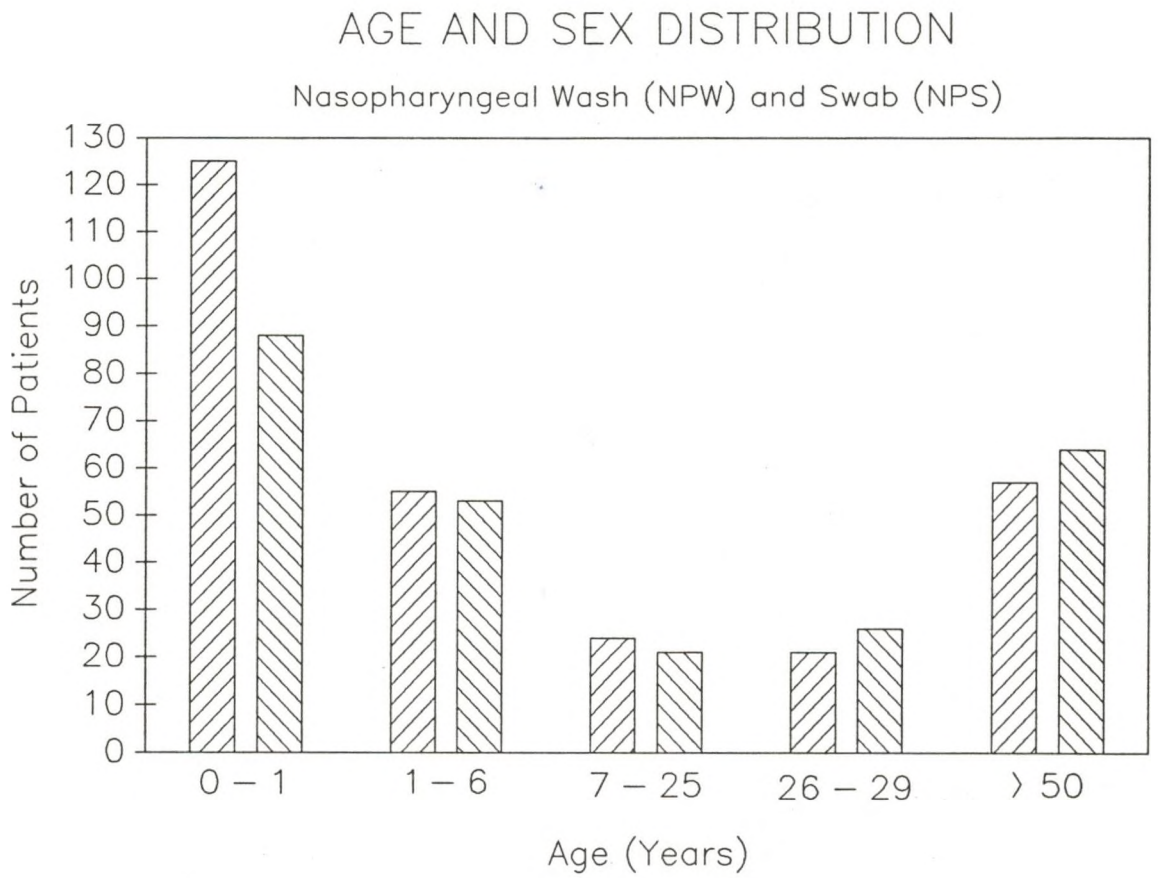
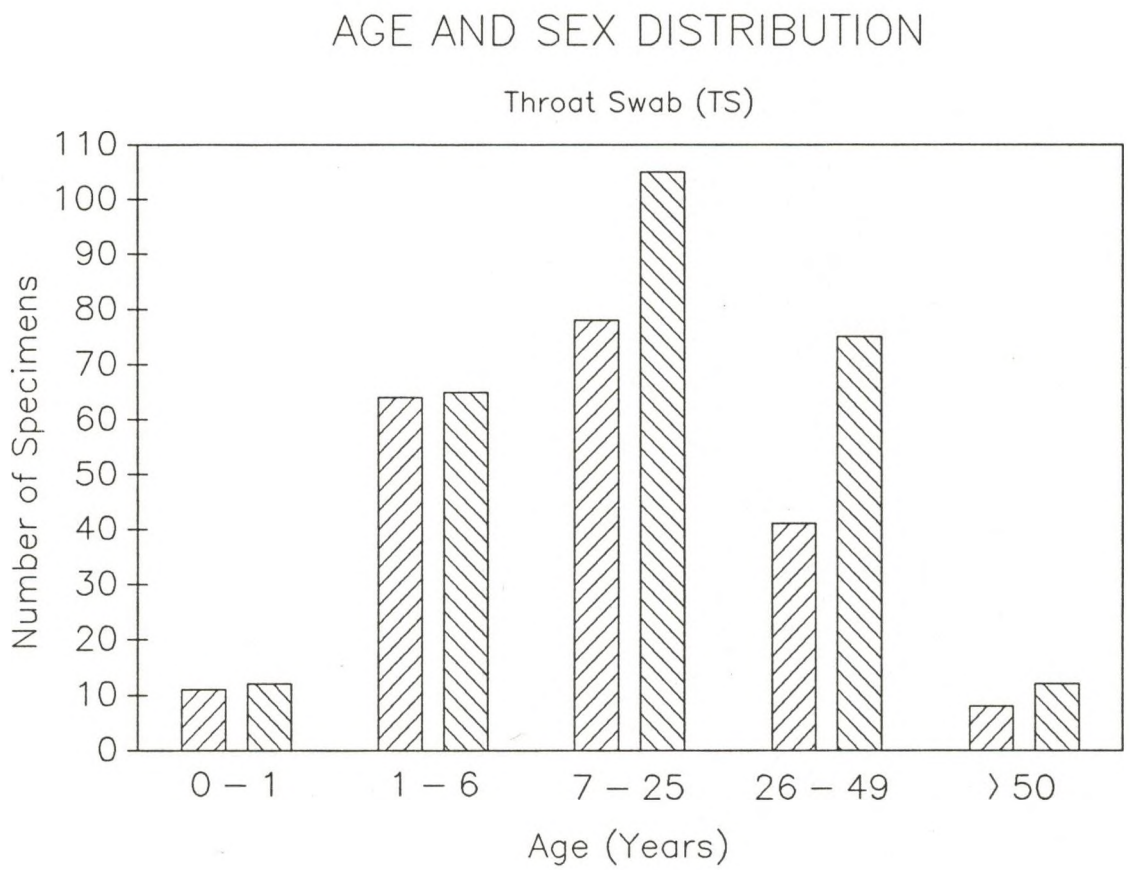

AGE AND SEX DISTRIBUTION
Nasopharyngeal Wash (NPW) and Swab (NPS)
Number of Patients
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0 – 1
1 – 6
7 – 25
26 – 29
› 50
Age (Years)

Figure 3. Age and sex distribution of patients from whom throat swabs were obtained. Symbols: Males, (///); Females, (\\\\).


AGE AND SEX DISTRIBUTION
Throat Swab (TS)
Number of Specimens
110
100
90
80
70
60
50
40
30
20
10
0
0 – 1
1 – 6
7 – 25
26 – 49
〉50
Age (Years)

were examined also for respiratory viruses (Table 5). Viruses and/or GAS were isolated from TS each month during the study period. Of the 480 specimens examined, 25.4% were positive for GAS and 7.7% were positive for respiratory virus (Table 5). In only 4 instances were GAS present when a virus was isolated from the same swab. The virus isolation rate was 8.9% in the GAS-negative specimens and 3.3% in the GAS-positive specimens. Seven groups of viruses were found with influenza A virus having the highest frequency of isolation (Table 6).

Identification of Viruses from Nasopharyngeal Swab and Nasopharyngeal Wash Specimens

In both respiratory disease years, the identification of viruses from NPS/NPW specimens was confined to the months of December through June. Peak viral identification rates occurred in February of 1988 and in May of 1989 (Table 7). An overall identification rate of 35.6% and a rate range of 0 to 56% were observed. The most frequently identified virus was RSV and the majority of the identifications were made from NPW specimens (Table 8). However, only a slight diagnostic advantage was seen for NPW specimens; 114 identifications were made from 290 NPW (39.3%) and 74 identifications were made from 237 NPS (31.2%). Eight of the viruses identified (5 herpes simplex viruses and 3 enteroviruses) were not screened for in the immunofluorescent staining procedure.

Table 5. Isolation of group A streptococcus and viruses from throat swab (TS) specimens.

Month/Year	Number Specimens	Group A Streptococcus Number Isolated	%	Viruses Number Isolated	%
12/87	15	1	7	2	13
1/88	50	15	30	9	18
2/88	20	3	15	1	5
3/88	33	12	36	6	18
4/88	52	13	25	1	2
5/88	52	12	23	2	4
6/88	43	6	14	2	5
7/88	0				
8/88	14	4	29	4	29
9/88	0				
10/88	40	6	15	3	8
11/88	38	12	32	1	3
12/88	42	15	38	1	2
1/89	2	1	50	1	50
2/89	10	3	30	3	30
3/89	52	14	26	2	4
4/89	17	5	29	0	0
Totals	480	122	25.4	38	7.7

Table 6. Comparative isolation frequency of viruses in throat swab specimens.

Virus	Number Isolated	Percent of Total
Influenza A	12	32.4
Enterovirus	7	18.8
Influenza B	5	13.8
Adenovirus	4	10.7
Herpes simplex	3	8.1
Parainfluenza type 1	2	5.4
Parainfluenza type 2	1	2.7
Parainfluenza type 3	1	2.7
Rhinovirus	1	2.7
Respiratory syncytial	1	2.7
Totals	37	100.0

Table 7. Identification of viruses from nasopharyngeal specimens.

Month/Year	Number of Specimens	Number of Viruses Identified(a)	Percent
11/87	4	0	0
12/87	17	1	5.9
1/88	37	14	37.8
2/88	50	28	56.0
3/88	49	19	38.8
4/88	20	6	30.0
5/88	2	1	50.0
6/88	No Tests		
7/88	No Tests		
8/88	No Tests		
9/88	No Tests		
10/88	1	0	0
11/88	5	0	0
12/88	25	6	24.0
1/89	50	17	34.0
2/89	83	20	24.1
3/89	58	18	31.0
4/89	37	11	29.7
5/89	63	36	51.1
6/89	26	12	46.2
Totals	527	189	

(a) - Includes immunofluorescent detection of viruses in direct patient specimens and isolation of viruses in cell culture.

Table 8. Comparative identification frequency of viruses in nasopharyngeal swab (NPS) and nasopharyngeal wash (NPW) specimens.

Virus	Identifications NPS	NPW	Total	Percent
Respiratory syncytial	34	72	106	55.9
Influenza A	6	18	24	12.8
Influenza B	16	7	23	12.2
Parainfluenza type 3	6	10	16	8.5
Adenovirus	6	4	10	5.3
Herpes simplex	4	1	5	2.7
Enterovirus	2	1	3	1.5
Parainfluenza type 1	0	2	2	1.1
Totals	74	115	189	100.0

Viral Incidence

In order to optimize the cost effectiveness of immunofluorescent staining methods, epidemiologic information concerning the incidence of respiratory viruses in the community must be known. This allows for the proper selection of reagents for use in routine staining procedures for respiratory specimens obtained at various times of the year. The seasonality and incidence of respiratory viruses in the Bismarck, North Dakota area are depicted in Figure 4 and Table 9, respectively. The seasonality data suggest that respiratory syncytial virus (RSV), influenza A and influenza B reagents could be excluded from testing from July through November. However, the endemic nature of adenoviruses and parainfluenza viruses necessitated that reagents for these viruses be included in staining procedures at all times of the year. Patients less than 6 years of age and those more than 50 years of age had the highest viral attack rates; almost half of the patients less than 12 months of age showed immunofluorescent or culture evidence of viral infection. While RSV was the most frequently encountered virus in the less than 6 year old group, influenza A virus was the most frequently identified virus in the remaining 4 age groups.

Viral Syndromes

Patients in the study population exhibited a wide variety of diagnoses and symptoms (Table 10). By ranking the most frequently occurring symptoms of patients with disease of known viral etiology,

Figure 4. Seasonality of respiratory viruses in Bismarck, North Dakota from December, 1987 to June, 1989.

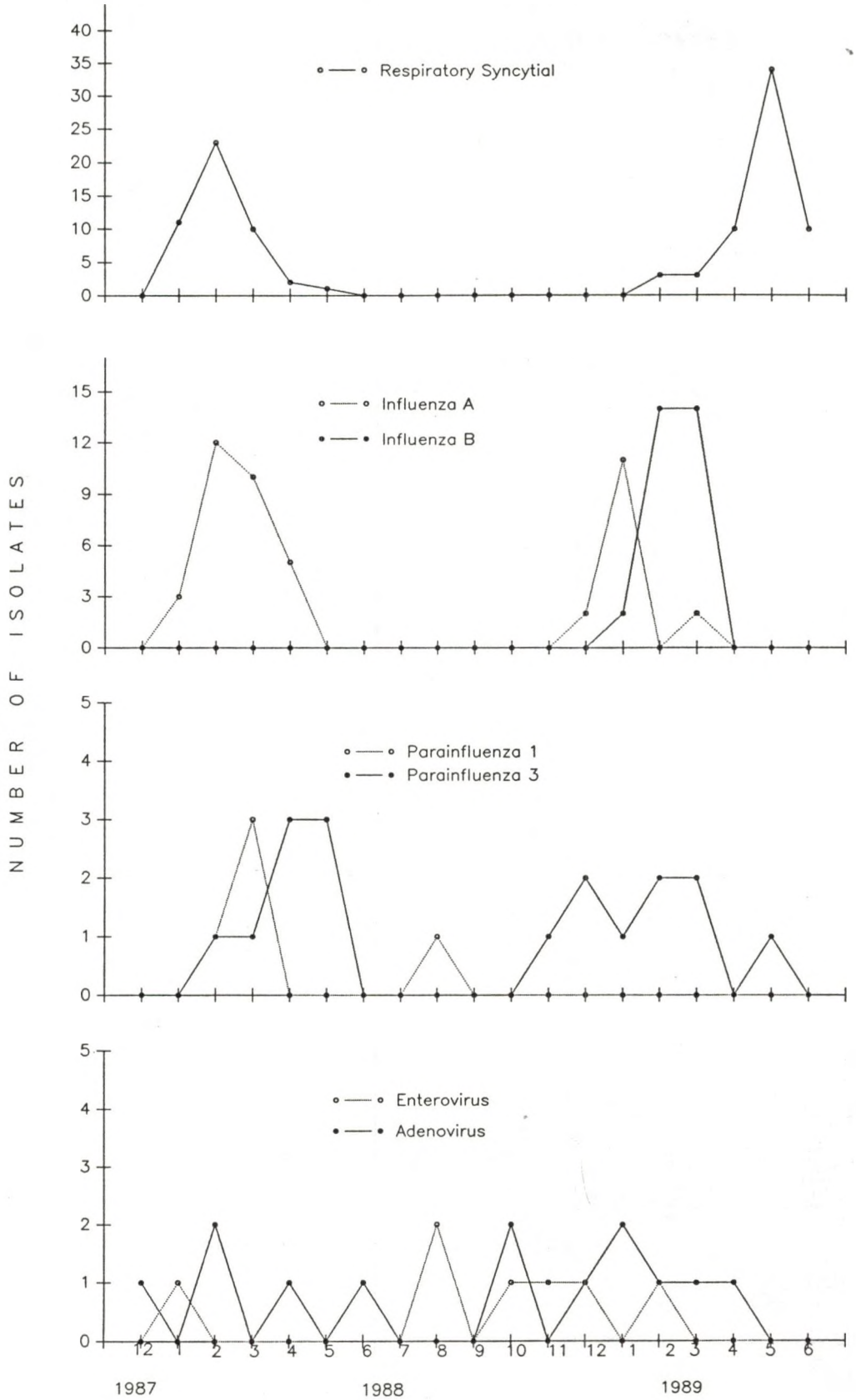

Respiratory Syncytial
Influenza A
Influenza B
Parainfluenza 1
Parainfluenza 3
Enterovirus
Adenovirus
NUMBER OF ISOLATES
0
5
10
15
20
25
30
35
40
3
6
9
12
1
2
4
12 1 2 3 4 5 6 7 8 9 10 11 12 1 2 3 4 5 6
1987
1988
1989

Table 9. Incidence by age group of respiratory syncytial virus (RSV), influenza virus types A and B (INF), parainfluenza virus types 1, 2, and 3 (PARA), and adenovirus (ADENO) in the Bismarck, North Dakota area.

Age Group	No.(a)	VIRUS IDENTIFICATIONS(b) RSV	INF	PARA	ADENO	Incidence Per Age Group (c)
≤ 1	229	81 (35.4)	9 (3.9)	11 (4.8)	6 (2.6)	107 (46.7)
2-6	239	20 (8.4)	21 (8.8)	7 (2.9)	7 (2.9)	55 (23.0)
7-25	229	1 (0.4)	15 (6.6)	2 (0.9)	1 (0.4)	19 (8.3)
26-49	166	0	5 (3.0)	0	0	5 (3.0)
≥ 50	144	4 (2.8)	14 (9.7)	2 (1.4)	0	20 (13.9)
Total	1007	106 (10.5)	64 (6.4)	22 (2.2)	14 (1.4)	Specific Viral Incidence (d)
						206 (20.5) Overall Viral Incidence (e)

(a) Number of specimens in each age group.
(b) Number and percent of specific viruses identified for specific age groups.
(c) Total number and percent of all viruses identified for specific age groups.
(d) Total number and percent of specific viruses identified for all age groups.
(e) Total number and percent of all viruses identified for all age groups.

Table 10. The diagnoses and symptoms, in decreasing order of frequency,of patients presenting with respiratory illness.

Diagnosis	Symptom
Pneumonia	Cough
Bronchitis	Fever
Upper Respiratory Infection	Pharyngitis
"Flu"	Rhinorrhea
Bronchiolitis	Respiratory Distress/ Shortness of Breath
Fever of Unknown Origin	Otitis
Asthma	Malaise
Pharyngitis	Wheezing
Chronic Cough	Vomiting
Pneumonitis	Congestion

viral syndromes were constructed (Table 11). Knowledge of these syndromes, in conjunction with known viral incidence in the community, could be useful to the physician for establishing etiology of disease and for directing decisions concerning therapy and diagnostic procedures.

Assessment of Specimen Quality for Immunofluorescent Assay of Direct Patient Specimens (DPS)

Specimens containing $\geq$2 ciliated epithelial cells per 400X field were judged to be acceptable for immunofluorescent assay (Figure 5). Overall, 46 (8.7%) of the nasopharyngeal specimens were unacceptable: 34 of 290 NPW specimens and 12 of 237 NPS specimens (Table 12). Age had a significant effect on specimen quality. The rejection rate for specimens from patients greater than 7 years of age was approximately 5 to 6 times higher than the rate for patients less than 6 years of age.

Interpretation of Immunofluorescent Staining Patterns in Virus-Infected Ciliated Epithelial Cells

Direct patient specimens were considered positive only when $\geq$ 2 cells per well had specific intracellular fluorescence. Typical immunofluorescence patterns were observed with all BIFA and BDFA-RSV reagents (Figure 6): cytoplasmic fluorescence with RSV and parainfluenza reagents, nuclear fluorescence with the influenza A and B reagents, and both nuclear and cytoplasmic fluorescence with the

Table 11. Syndromes associated with specific viral infections, listed in decreasing order of frequency.

Virus	Syndrome
Respiratory syncytial	Cough > Fever > Rhinorrhea > Wheezing
Influenza	Fever > Cough > Pharyngitis > Malaise
Parainfluenza type 3	Cough > Wheezing > Fever > Otitis
Adenovirus	Fever > Otitis > Cough > Diarrhea
Herpes simplex	Pharyngitis > Cough > Fever > Respiratory distress
Enterovirus	Fever > Pharyngitis > Cough > Headache

Figure 5. A direct patient specimen (DPS) containing $\geq$ 2 ciliated epithelial cells (CEC) per 400X field.

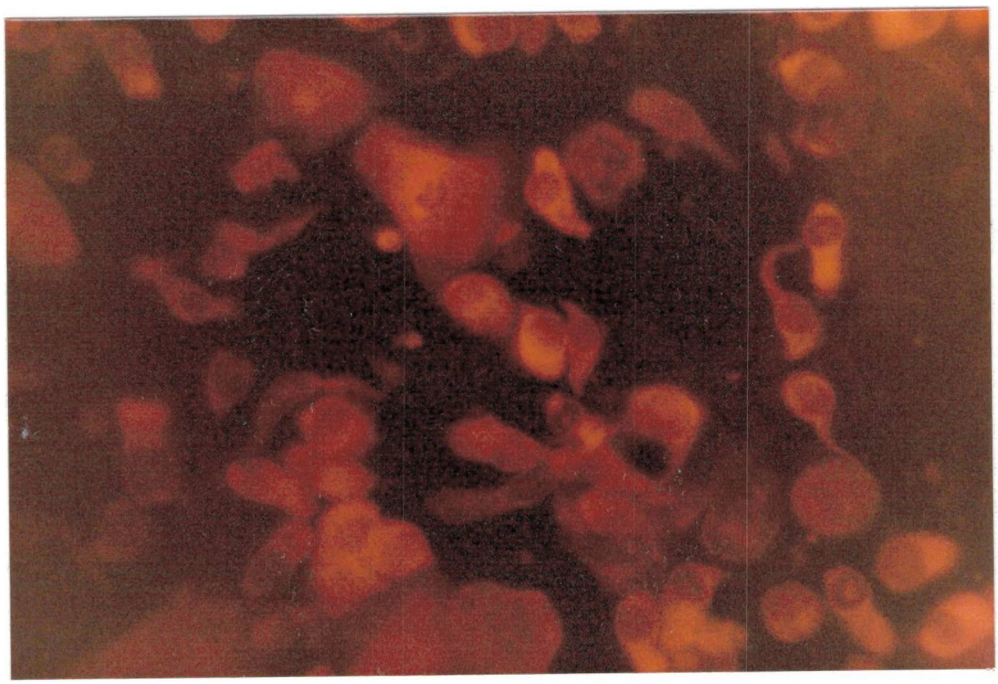
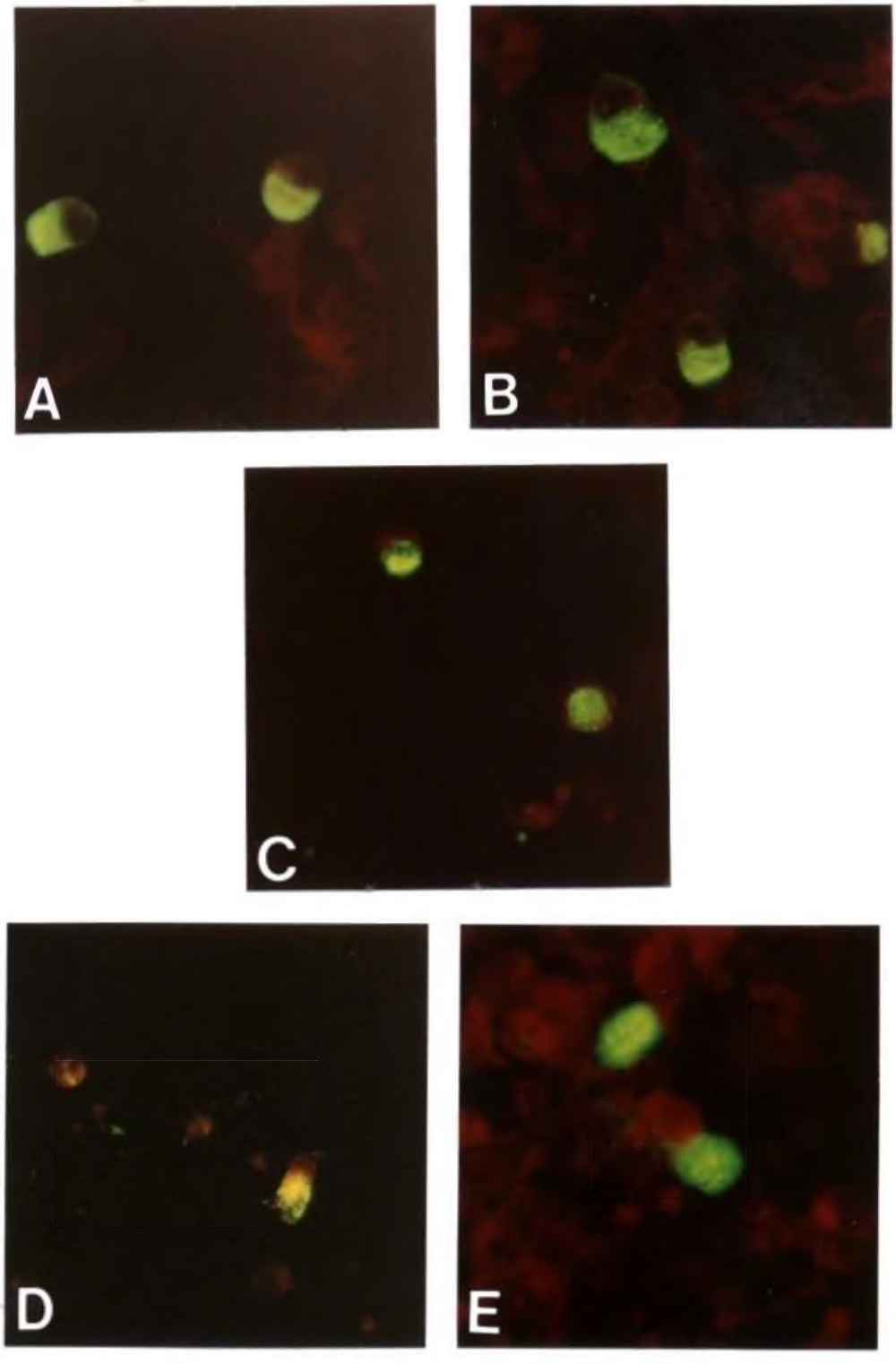


Table 12. The quality of nasopharyngeal swab (NPS) and nasopharyngeal wash (NPW) specimens with respect to age.

	NPS			NPW		
Age	Total Specimens	Not Acceptable	%	Total Specimens	Not Acceptable	%
All	237	12	5.1	290	34	11.7
≤ 6 Years	116	2	1.7	201	9	4.5
≥ 7 Years	121	10	8.3	89	25	28.1

Figure 6. Bartels IFA staining of virus-infected ciliated epithelial cells (400X). (a, BDFA-RSV; b, BIFA-RSV; c, BIFA-FA; d, BIFA-P3; and e, BIFA-Ad).


A
B
C
D
E

adenovirus reagent. Immunofluorescent patterns observed with WDFA reagents were typical for RSV, parainfluenza, influenza A and adenovirus (Figure 7). Influenza B virus-infected cells displayed primarily cytoplasmic fluorescence that had a tendency to intensify at the brush border (Figure 7c). Information concerning the viral epitope against which the monoclonal antibodies were directed was either unknown or unavailable from the manufacturers.

Nonspecific background fluorescence was observed in approximately 10% of the specimens which correlated with increased numbers of squamous epithelial cells and mucus in these specimens (Figure 8).

Efficiency of Immunofluorescence for the Detection of Respiratory Viruses in Direct Patient Specimens

The sensitivity, specificity and predictive value of each immunofluorescent staining method and of each individual reagent for all subsequent experiments were calculated as previously described. No conclusions were made concerning reagents for parainfluenza virus type 1 and 2 because of the low number of isolates.

Comparative Bartels Indirect Fluorescent Antibody (BIFA) and Culture Data

The BIFA procedure resulted in various levels of sensitivity and specificity (Figure 9 and Table 13). The sensitivity of the influenza B, parainfluenza 3 and adenovirus reagents (55.6%, 66.7%, and

Figure 7. Whittaker DFA staining of virus-infected ciliated epithelial cells (400X). (a, WDFA-RSV; b, WDFA-FA; c, WDFA-FB; d, WDFA-P3; and e, WDFA-Ad).

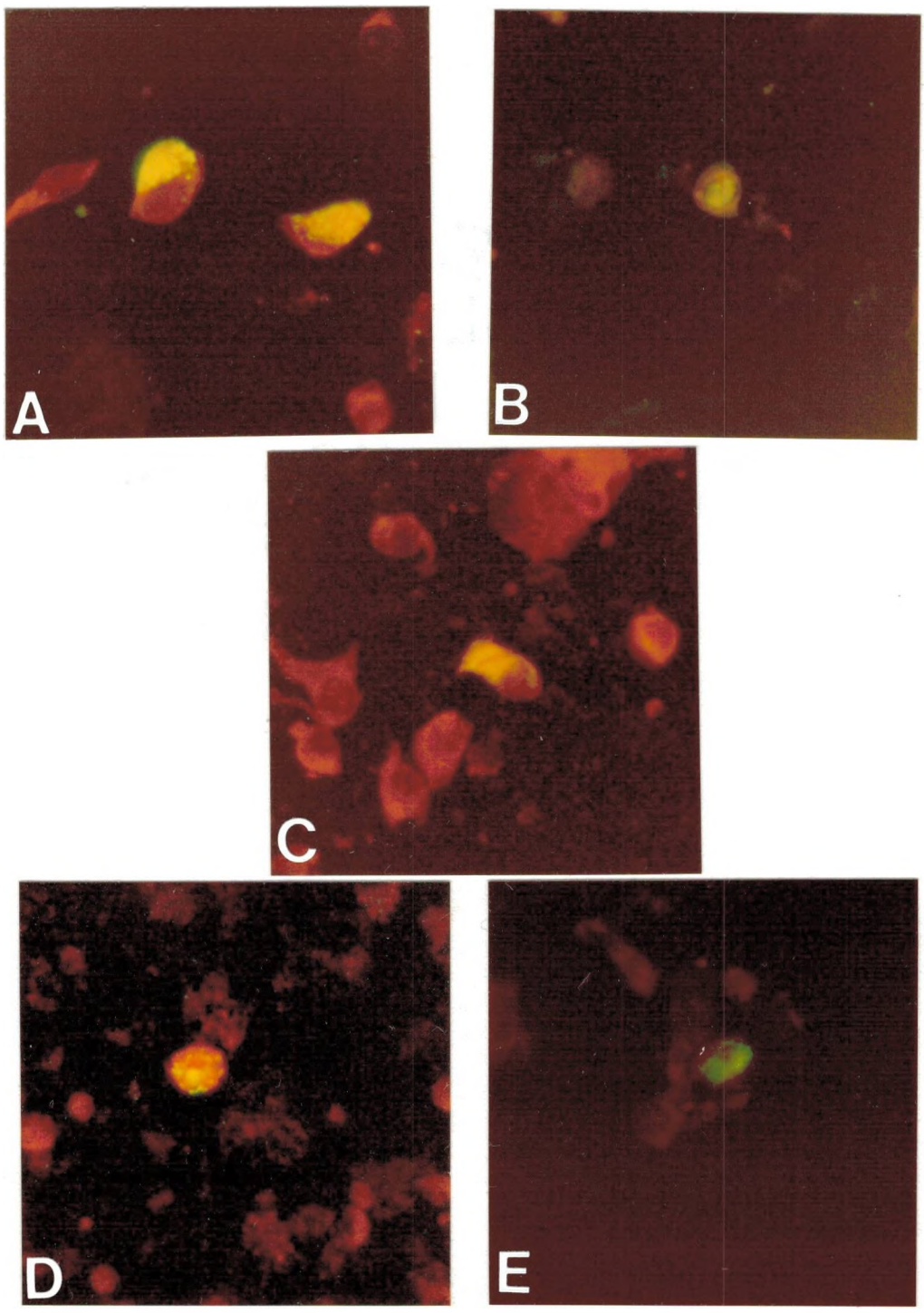

A
B
C
D
E

Figure 8. Nonspecific staining of a direct patient specimen (Bartels IFA pool reagent, 400X).

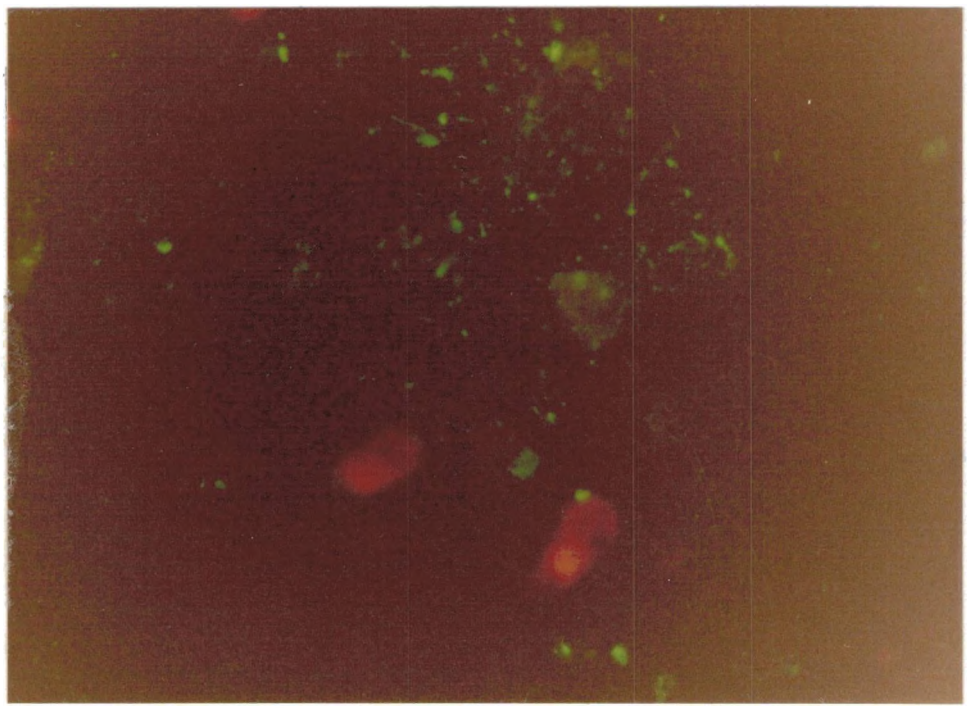


Figure 9. Comparative Bartels indirect fluorescent antibody (BIFA) and culture data.

		BIFA	
		POSITIVE	NEGATIVE
CULTURE	POSITIVE	Respiratory Syncytial -48 Influenza A -13 Influenza B - 5 Parainfluenza type 3 - 8 Adenovirus - 3 Total -77	Influenza B - 4 Parainfluenza type 3 - 4 Adenovirus - 6 Total -14
	NEGATIVE	Respiratory Syncytial -35 Influenza A - 3 Influenza B -14 Parainfluenza type 3 - 3 Total -55	Total -274

Table 13. Efficiency of the Bartels indirect fluorescent antibody reagents.

Virus	Sensitivity (%)	Specificity (%)	PPV (%)*	NPV (%)**
Respiratory syncytial	100	87.3	57.8	100
Influenza A	100	98.9	81.3	100
Influenza B	55.6	95.1	26.3	98.6
Parainfluenza type 3	66.7	98.9	72.7	98.6
Adenovirus	33.3	100	100	97.9
Overall	84.6	83.3	58.3	95.1

* - Positive predictive value
** - Negative predictive value

50%, respectively) lowered the overall sensitivity of the method to 84.6%. The specificities of the individual reagents were excellent, however, the higher number of false positive RSV and influenza B reactions resulted in an overall specificity of 83.3%. The lack of specificity of the RSV reagent was questionable since evidence suggests that immunofluorescence is more specific than cell culture for detection of RSV (63). The positive and negative predictive values of the test were 58.3% and 95.1%, respectively. Table 14 demonstrates the effects of viral prevalence on the predictive values of the BIFA procedure. At low prevalence, these reagents had very high predictive values for a negative test but very low predictive values for a positive test. These data suggest that at low prevalence, a negative result is useful for deciding on the absence of disease. As prevalence increases to 50%, the predictive values for a positive test improve significantly, enhancing the value of a positive test to confirm the presence of disease.

Comparative Whittaker M. A. Bioproducts Direct Fluorescent Antibody (WDFA) and Culture Data

High specificity and low sensitivity percentages were obtained with the WDFA method (Figure 10 and Table 15). Only the influenza A reagent was satisfactory while the poorer results with the remaining reagents lowered the overall sensitivity to 50%. The positive predictive value of this procedure was 53.0% and the negative predictive value was 91.0%. The effect of varying prevalence rates on the predictive values of the WDFA reagents is presented in Table 16. The

Table 14. Effect of varying viral prevalence on predictive values of the Bartels indirect fluorescent antibody reagents.

Virus	PREVALENCE (%) 1	5	10	50
Respiratory syncytial				
PVP*	7.4	29.3	46.7	88.7
PVN**	100	100	100	100
Influenza A				
PVP	47.9	82.7	91.0	98.9
PVN	100	100	100	100
Influenza B				
PVP	10.3	37.4	55.8	91.9
PVN	99.5	97.6	95.1	68.2
Parainfluenza type 3				
PVP	23.4	61.4	77.1	96.8
PVN	99.7	98.3	96.4	74.8
Adenovirus				
PVP	100	100	100	100
PVN	99.3	98.7	96.6	60.0
Overall				
PVP	4.6	21.1	36.0	83.5
PVN	99.8	99.1	98.0	84.6

* - Predictive value of a positive test, in percent.
** - Predictive value of a negative test, in percent.

Figure 10. Comparative Whittaker direct fluorescent antibody (WDFA) and culture data.

CULTURE	WDFA: POSITIVE	WDFA: NEGATIVE
POSITIVE	Respiratory Syncytial - 8 Influenza A -16 Influenza B - 4 Parainfluenza type 3 - 2 Adenovirus - 5 Total -35	Respiratory Syncytial -13 Influenza A - 1 Influenza B - 5 Parainfluenza type 3 - 9 Adenovirus - 5 Total -33
NEGATIVE	Respiratory Syncytial -16 Influenza A - 4 Influenza B -11 Total -31	Total (Respiratory syncytial only) -240 Total -332

Table 15. Efficiencies of Whittaker direct fluorescent antibody reagents.

Virus	Sensitivity (%)	Specificity (%)	PPV (%)*	NPV (%)**
Respiratory syncytial	38.1	93.8	33.3	94.9
Influenza A	94.1	98.8	80.0	99.7
Influenza B	44.4	96.8	50.0	98.5
Parainfluenza type 3	18.2	100	100	97.1
Adenovirus	50.0	100	100	98.7
Overall	50.0	91.5	53.0	91.0

* - Positive predictive value
** - Negative predictive value

Table 16. Effect of varying viral prevalence on predictive values of the Whittaker direct fluorescent antibody reagents.

Virus	PREVALENCE (%) 1	5	10	50
Respiratory syncytial				
PVP*	5.8	24.4	40.6	86.0
PVN**	99.4	96.6	93.2	60.2
Influenza A				
PVP	44.5	80.5	89.7	98.7
PVN	99.9	99.7	99.3	94.4
Influenza B				
PVP	12.3	42.2	60.7	93.3
PVN	99.4	97.1	94.0	63.5
Parainfluenza type 3				
PVP	100	100	100	100
PVN	99.1	95.9	91.7	55.0
Adenovirus				
PVP	100	100	100	100
PVN	99.5	97.4	94.7	66.7
Overall				
PVP	5.6	23.6	39.5	79.7
PVN	99.0	94.8	94.3	64.7

* - Predictive value of a positive test, in percent.
** - Predictive value of a negative test, in percent.

100% specificity of the parainfluenza type 3 and adenovirus reagents account for the 100% positive predictive values. As with the BIFA reagents, the WDFA procedure demonstrated the highest negative predictive values at low prevalence and the highest positive predictive values at high prevalence.

Comparative Bartels Anti-Respiratory Syncytial Virus Direct Fluorescent Antibody (BDFA-RSV) and Culture Data

The efficiency and predictive values of the BDFA-RSV reagent compares favorably with the BIFA-RSV reagent (sensitivity 100%, specificity 89.1%, positive predictive value 63.2% and negative predictive value 100%) (Figure 11). These data were not surprising because the monoclonal antibody used in the BDFA-RSV reagent was the same antibody used in the BIFA-RSV and BIFA pool reagents.

Efficiency of Shell Vial (SV) Assay for Isolation of Viruses from Clinical Specimens

Comparative HEp-2 Shell Vial (SV) Assay and Tube Culture Data

Shell vials seeded with HEp-2 cells and stained at 48 hours with the BDFA-RSV reagent appeared to be superior to HEp-2 tube cell culture for the isolation of RSV (Figure 12). Eleven RSV isolations occurred only in the SV system whereas 1 isolation occurred only in the

Figure 11. Comparative Bartels anti-respiratory syncytial virus direct fluorescent antibody (BDFA-RSV) and culture data.

		BDFA-RSV	
		POSITIVE	NEGATIVE
CULTURE	POSITIVE	67	0
	NEGATIVE	39	318

Figure 12. Comparison of HEp-2-seeded shell vials and conventional tube cultures for the isolation of respiratory syncytial virus.

		HEp-2 SHELL VIAL	
		POSITIVE	NEGATIVE
CULTURE	POSITIVE	15	1
	NEGATIVE	11	37

tube culture. Isolation of RSV in SV also compared favorable with BDFA detection of RSV-infected cells in direct patient specimens (Figure 13).

Comparative A549 Shell Vial (SV) Assay and Tube Cell Culture Data

Shell vials seeded with A549 cells and stained at 48 hours with the BIFA pool reagent failed to isolate 18 viruses that were isolated by tube cell culture (Figure 14). Conversely, 1 virus was isolated only in the A549 SV assay. Detection of virus-infected cells in direct patient specimens by BIFA was also superior to isolation of the viruses in A549 SV (Figure 15).

Figure 13. Comparative data for the isolation of respiratory syncytial virus (RSV) in HEp-2-seeded shell vials and detection of RSV-infected cells in direct patient specimens by Bartels anti-RSV direct fluorescent antibody (BDFA-RSV) reagent.

		HEp-2 SHELL VIAL	
		POSITIVE	NEGATIVE
BDFA-RSV	POSITIVE	25	5
	NEGATIVE	0	34

Figure 14. Comparison of A549-seeded shell vials and conventional tube cell culture for the isolation of respiratory viruses.

A549 SHELL VIAL

		POSITIVE	NEGATIVE
CULTURE	POSITIVE	2	18
	NEGATIVE	1	88

Figure 15. Comparative data for the isolation of respiratory viruses in A549 shell vials and the detection of virus-infected cells in direct patients specimens by Bartels indirect fluorescent antibody (BIFA).

A549 SHELL VIAL

BIFA	POSITIVE	NEGATIVE
POSITIVE	1	24
NEGATIVE	2	82

DISCUSSION

Although viral diseases are the most common human infections, it is paradoxical that most microbiology laboratories do not provide viral diagnostic services. This diagnostic deficiency has been exacerbated by the advent of specific antiviral therapy. The genuine need for rapid diagnosis coupled with the development of monoclonal antibodies has revived immunofluorescence as the a priori method of rapid viral diagnosis. Our objectives were to evaluate the efficiency of new monoclonal antibodies to detect respiratory virus antigens in clinical specimens and to determine the usefulness of this technique in a community hospital laboratory.

Immunofluorescent reagents were utilized in this study to detect viral antigens in direct patient specimens and to stain specimen-inoculated cell cultures for virus identification. This dual diagnostic role broadened the utility of immunofluorescence by providing a powerful tool for studying viral epidemiology. The virologic study of respiratory specimens collected from hospitalized and outpatient respiratory specimens provided a good model for virus surveillance. Our data demonstrate that throat swabs, submitted for isolation of group A streptococcus (GAS), could be effectively used for virus isolation. The isolation rates for viruses and GAS from throat swab (TS) specimens were 25% and 7.7%, respectively, and compare favorably with a previous study (87). Conversely, 67.3% of the TS specimens were negative for both

viruses and GAS. Since the majority of the patients from whom TS specimens were obtained consisted of older children and young to middle-aged adults, it is possible that Mycoplasma pneumoniae, and noncultivatable viruses such as coronavirus and Epstein-Barr virus caused significant morbidity in the culture-negative TS population. In studies of acute respiratory syndromes in young adults, one third to one half of the causes remain unidentified (2). Four instances of concomitant virus and GAS infection were identified, including one isolate each of influenza A, parainfluenza type 1, adenovirus, and enterovirus. The concomitant isolation rate of 0.8% is lower than that reported in similar studies (87,88) and the number of different viruses isolated in conjunction with GAS suggest that there is no apparent tendency for one viral infection to predispose more than another to such an association. The low concomitant infection rate is surprising since most of the TS specimens were collected in the winter and spring months during periods of high respiratory virus and GAS transmission in the community. A major complication of viral respiratory infections is bacterial superinfection and the organisms most often associated with this condition include Streptococcus pneumoniae, Haemophilus influenzae, and Staphylococcus aureus (89). That superinfection does not occur frequently with GAS is suggested in this study not only by the low rate of concomitant GAS infections but also by a virus isolation rate that was nearly 3 times higher in the GAS-negative specimens as compared to the GAS-positive specimens. These data may conflict with the results of a study of pharyngitis and tonsillitis among college students (88). These researchers indicated that virus infection may predispose to

streptococcal infection (or vice versa) because 27% of the virus-positive patients were concomitantly infected with GAS while the streptococcal carrier rate in patients without disease was only 8%.

The comparative virus isolation frequencies from TS and nasopharyngeal (NP) specimens are compatible with the age distribution of the patients. Influenza viruses accounted for nearly half of the virus isolations in the TS group and for 25% of the isolations in the NP group. These isolation rates are proportional to the number of patients of day care and school age in each specimen population and are consistent with the belief that these children disseminate influenza in the community and introduce infection into families (2). The total number of respiratory syncytial virus (RSV) and parainfluenza virus (PIV) isolations correlated well with the number of infants and young children in each specimen population. Thirty-three percent of the TS specimens and 61% of the NP specimens were obtained from patients less than 6 years of age and the respective RSV/PIV isolation rates were 13.5% and 65.5%.

Eight cultures were positive for herpes simplex virus (HSV) which accounted for 8.3% and 2.7% of the virus isolates for TS and NP specimens, respectively. Evans and Dick proposed that HSV may cause acute tonsillitis or pharyngitis, especially in older children and young adults (88). Our data support this finding because 3 of the 8 HSV-positive patients (ages 1, 16, and 22 years) had pharyngitis without the presence of oral lesions. The role of HSV as an etiologic agent of respiratory disease was obscured in the remaining patients by the presence of gingival, labial or buccal lesions.

Pharyngitis was the primary symptom of patients suffering from enteroviral infection. Two of the 10 enteroviruses isolated were identified as coxsackie B4 and echo 2. Although the etiology was not proven by neutralization tests performed with the patient's acute and convalescent sera, the presence of these viruses in the pharynx suggests that they may have contributed to the clinical picture and support the contention that pharyngitis is a clinical manifestation of coxsackievirus and echovirus infections (45).

Adenovirus isolation rates were 10.8% in TS specimens and 5.3% in NP specimens. Eleven of the 14 adenovirus-positive patients were infants less than 2 years of age and the majority of these children had acute upper respiratory tract symptoms including cough, fever, and otitis media. Although these virus isolates were not typed, our data are consistent with Glezen and Denny who concluded that adenovirus types 1, 2, and 5 have been correlated best with the occurrence of febrile upper respiratory illnesses in infants under 2 years of age.

A more complete picture of the incidence of respiratory viruses in the Bismarck, North Dakota area was provided by combining the TS and NP specimen data. Infants, young children and adults greater than 50 years of age had the highest incidence of viral infection; 46.7%, 23.0%, and 13.9%, respectively. Respiratory syncytial and parainfluenza viruses were most frequently identified in infants, whereas influenza viruses and RSV were the most commonly identified agents in young children and adults over 50 years of age. The importance of RSV as the major viral pathogen of infancy and early childhood was supported by a 43.8% incidence of RSV in children less than 6 years of age and by the finding

that 30.2% of the RSV isolates were recovered from infants hospitalized with severe lower respiratory tract disease. Of 32 infants hospitalized with RSV infections, 21 (66%) were less than 6 months of age, demonstrating the unique ability of RSV to cause serious lower respiratory tract disease even in the presence of maternal antibody (6). Two of the infants and one elderly patient developed lower respiratory tract disease serious enough to require treatment with ribavirin. Although approximately one-third of the hospitalized infants were premature at birth, no evidence of deficient cellular response could be found in the medical histories which could account for enhanced morbidity in this population.

Reinfection with RSV and PIV occurs with appreciable frequency in older children and adults and probably plays a major role in the spread of virus to the young infant (2). Our data show little evidence to support this statement. However, RSV and PIV infections in older children and adults cause mild upper respiratory illnesses which generally would not result in a physician visitation. Because the only participants included in this study were patients exhibiting respiratory illness serious enough to seek medical attention, it is probable that the numbers of RSV and PIV infections in older children and adults were underestimated.

Although influenza viruses caused significant illness in each age group, the highest incidence of influenza virus infection was in patients greater than 50 years of age. An estimate of influenza morbidity was made by calculating the percentage of patients hospitalized in each age group: infants, 38%; young children, 33%;

older children and young adults, 33%; adults 26 to 49 years of age, 40%; and adults greater than 50 years of age, 79%. These data indicate that an increased morbidity occurs in elderly patients which is corroborative of previous reports (3,13). Welliver and Ogra have speculated that this increased morbidity in the elderly is due to a waning of immunity related to diminished proliferative responses and decreased interleukin-2 production by cytotoxic T-lymphocytes (6).

Although the vast majority of respiratory infections occurred in patients less than 6 years of age, adults greater than 50 years of age had an appreciable number of RSV and PIV infections, reinforcing the fact that these viruses cause illness throughout life and that a closer surveillance of our elderly population is warranted. The overall virus identification rate of 20.5% emphasizes the scope of viral infections in the community and explains the heavy burden of morbidity due to viral respiratory illness.

Epidemics of RSV were observed in both years of the study. In 1987-88, the epidemic began sharply in January and peaked in February and the RSV epidemic of 1988-89 had a gradual increase of cases beginning in February and a peak incidence in May and June. Although RSV was predictably epidemic in both years, the peak incidence shifted from February of the first year to May of the second year. This finding is consistent with previous studies that indicated epidemics of RSV disease occurred yearly but they alternated in their occurrence between mid-winter and early spring (3,25,27). Epidemics of influenza A virus were seen in both respiratory disease years; peak influenza A (H1N1) activity occurred in February of 1988 and the greatest incidence of

influenza A (H3N2) was seen in January of 1989. Although not observed during 1988, an epidemic of influenza B occurred in 1989 with peak incidence observed in the months of February and March. A sharp demarcation was seen between influenza A and B epidemics; only 1 additional isolation of influenza A was obtained after the first isolation of influenza B had been made. The majority of parainfluenza and adenovirus isolates were detected from December through April of both respiratory years, with sporadic isolates of both viruses encountered during the summer and fall. Adenoviruses were endemic with at least one infection occurring in 10 out of the 18 months of the study.

Glezen and Denny observed a type of interference phenomenon that appeared to influence the occurrence of lower respiratory tract infections with the major respiratory viruses (25). In general, when the peak incidence of one major virus occurred, the other major viruses were absent. Our epidemiologic data tend to substantiate their observation, especially in the second year of the study. In 1989, peak epidemic months for influenza A, influenza B, and RSV were observed to be January, February, and May, respectively, with very little overlap of isolations. Parainfluenza type 3 virus was co-circulating during all 5 months but at a very low level. Although RSV and influenza A virus both had a peak incidence in February of 1988, parainfluenza virus types 1 and 3 peaked in March and April of 1988, respectively.

A corollary to the interference phenomenon is the fact that the total illness rate did not vary significantly from year to year regardless of the number of types of respiratory viruses present. In

the first respiratory year of the study, a total of 106 infections were recognized whereas in the second year, 120 infections were observed. Even though an epidemic of influenza B did not occur during the first respiratory year, the total number of infections for each year was similar.

A necessary prerequisite to successful detection of respiratory virus antigens in clinical specimens by immunofluorescence was the collection of specimens with an adequate number of virus-infected ciliated epithelial cells (CEC). Both NPW and NPS specimens were evaluated for CEC-harvesting efficiency; 5.1% of NPS and 11.7% of NPW specimens were judged as unsuitable for immunofluorescent assay. In patients less than 6 years of age, the overall rejection rate was 3.5% with the likelihood of rejection nearly 3 times greater for NPW than for NPS. The influence of age on harvesting efficiency of CEC was evident by the 16.7% rejection rate in patients greater than 7 years of age. The probability of rejecting a NPW in this age group was more than 3 times greater than that of rejecting a NPS. Respiratory infections in adults characteristically lack mucus secretion and adults excrete much less virus antigen than children (80), thus making it difficult to obtain a good sample for antigen detection. The difficulty of successful collection of nasopharyngeal specimens in patients greater than 7 years of age could be related to amelioration of the infectious process by an experienced immune system resulting in much less desquamation and necrosis of the epithelial lining of the nasopharynx. Previous studies have reported rejection rates of 32% for nasopharyngeal suctions (81) and 1.8% for pooled NPS and TS (52). The 8.7% rejection

rate in this study was acceptable considering that specimens were collected from all age groups.

Approximately 20% of nasopharyngeal specimens in the study were pooled with TS. although the amount of nonspecific fluorescence was generally increased by this action, the recovery of specific viruses in cell culture was significantly improved. Of 10 NPS specimens that were positive for influenza B virus by immunofluorescence but negative by culture, none were supplemented by a TS specimen, whereas 5 of 6 NPS specimens positive for influenza B virus by immunofluorescence and culture were augmented by a TS.

The excellent sensitivity and specificity of the BIFA-RSV and BDFA-RSV reagents support earlier findings suggesting that immunofluorescence could replace cell culture as the routine method for diagnosis of RSV infection (52,84). The equivalent efficiencies demonstrated by these reagents corroborate studies that were unable to demonstrate any significant difference in results between direct and indirect immunofluorescence methods with respect to RSV detection (52,84). Although highly specific, the sensitivity of the WDFA-RSV reagent was inferior to the Bartels reagents.

The impressive performance of the BIFA-FA and WDFA-FA reagents suggest that either of these reagents could replace cell culture as the routine method for diagnosis of influenza A infections. The sensitivity and specificity of the BIFA-FA reagent (100% and 98.9%, respectively) and the WDFA-FA reagent (94.1% and 98.8%, respectively) are significantly better than efficiencies of monoclonal antibodies previously described (56,72).

The specificities of the influenza B, parainfluenza type 3 and adenovirus monoclonal antibodies of both manufacturers were excellent; however, the sensitivity of each reagent could be improved. These data are consistent with the low sensitivities of monoclonal antibodies reported in previous studies of influenza B (56) and adenovirus (80). The high specificities of the reagents allow for confident presumptive diagnoses.

Given the prevalence of disease and the sensitivity and specificity of the test, one can estimate the probability of disease from a test result. The predictive value of a positive test result is an estimate confirming the presence of disease while the predictive value of a negative test result is an estimate that disease is not present (55). At low prevalence, the BIFA and WDFA procedures demonstrated high negative predictive values and low positive predictive values indicating that a negative test result would be useful for ruling out the presence of disease and that a positive test result would have a low probability of detecting disease. At 50% prevalence, the predictive values of a positive test result for both the BIFA and WDFA procedures are high enough to be useful in confirming the presence of disease. One would have more confidence in ruling out the presence of disease with a negative BIFA result since the 84.6% negative predictive value for this procedure was significantly greater than the 64.7% negative predictive value of the WDFA procedure.

Overall, the BIFA reagents demonstrated a better balance of sensitivity and specificity than did the WDFA reagents. Generally, the WDFA reagents displayed less intense immunofluorescent staining of

infected cells than did the counterpart BIFA reagents. The immunofluorescent staining patterns of the WDFA-RSV and WDFA-FB reagents were judged to be of a duller and hazier quality as compared to the BIFA-RSV and BIFA-FB reagents.

A disturbing finding with both the DFA and IFA methodologies was the apparent high number of false positive results with respect to virus isolation in cell culture. Fulton and Middleton have demonstrated that immunofluorescence had a diagnostic advantage over cell culture isolation when specimens were taken in the late stages of infection (81). Other investigators have presented evidence suggesting that testing by immunofluorescent assays may result in positive results in samples taken up to one week after the onset of infection, when specimens obtained at the same time have become negative for virus by cell culture (57,90-92). The clinical histories of the patients whose specimens demonstrated false positive immunofluorescent results were reviewed to ascertain the time that the specimens were taken in relation to the onset of symptoms. In 14 cases (10 RSV and 4 influenza B diagnoses, respectively) the patients were symptomatic for 4 to 10 days prior to specimen collection. The phenomenon of immunofluorescent positive and culture negative results could be explained by the appearance of secretory antibodies which reduce the infectivity of free virus even though viral antigens may still be present in exfoliated epithelial cells.

The efficiency of conventional cell culture to isolate all viruses must be considered in the discussion of false positive immunofluorescent results. Since the immunofluorescent reagents were quality controlled

and specific intracellular patterns of fluorescence were observed, should one consider the immunofluorescent results falsely positive or the culture results falsely negative? Investigators have demonstrated the inefficiency of cell culture for isolation of RSV (91), influenza virus (13), parainfluenza viruses (90), and adenovirus (66). If reagents have been tried and tested and the reader is absolutely familiar with the appearance of specific virus fluorescence in clinical material, then it is possible to make a confident diagnosis by immunofluorescence alone (77).

Reconsideration of the false positive immunofluorescent results as true indicators of viral infection results in significant improvement in the diagnostic power of the BIFA and BDFA-RSV assays. Examination of the BIFA and culture comparative data indicates that 91 identifications could be made by culture alone and that 132 identifications could be made by immunofluorescence alone. Similarly, the 106 identifications made by the BDFA-RSV reagent would be significantly greater than the 67 RSV identifications made by cell culture alone. Review of the WDFA and cell culture comparative data indicates no significant difference between immunofluorescence and cell culture isolation in terms of the number of positive diagnoses made by each method alone (68 for cell culture versus 66 for immunofluorescence). For both the BIFA and BDFA-RSV reagents, the number of positive diagnoses that would have been made if immunofluorescence had been the only diagnostic method was significantly greater than the number that would have been made if only isolation in cell culture had been used.

Use of both immunofluorescence and cell culture resulted in the

greatest diagnostic efficiency. The 146 diagnoses made by the combination of BIFA and cell culture isolation was significantly greater than the 132 diagnoses made by immunofluorescence alone or the 91 diagnoses made by culture alone. Increased diagnostic efficiency was also observed with tandem use of WDFA reagents and cell culture isolation. A total of 99 diagnoses could be made by combining methods, whereas 31 diagnoses could be made by immunofluorescence alone and 33 diagnoses made by culture alone.

At the present time, rapid diagnosis of viral respiratory infections is limited to ELISA and immunofluorescent assay. In our hands, immunofluorescence was a cost effective and convenient method for viral diagnosis. The reagent costs for staining a DPS with the individual BIFA and WDFA reagents were approximately $1.79 and $1.24, respectively, which compares favorably with ELISA. The total time of testing, including incubations, is 45 minutes for DFA and 90 minutes for IFA. Depending upon the manufacturer, ELISA procedures usually can be performed in 2 to 3 hours.

A positive immunofluorescent result has the potential to reduce or eliminate the need for routine cell culture which is labor intensive and requires considerable clinical expertise. Minnich and Ray calculated the hands-on time for an individual IFA to be 18.3 minutes and for a routine cell culture with identification by neutralization to be 82.1 minutes (52). The difference of 64 minutes for each cell culture eliminated would result in significant savings in time and money for the virology laboratory. It should be noted that decisions concerning the deletion of cell culture based on rapid detection results must be made

with caution since viral agents other than those tested for by immunofluorescence may be present. Eight viruses that were isolated from NPW/NPS specimens could not have been detected with the immunofluorescent reagents used in the study.

The efficiency of shell vial assay for the detection of herpes simplex virus has been demonstrated in the laboratory at the Quain and Ramstad Clinic. Shell vials seeded with MRC-5 cells displayed a 90.9% sensitivity and a 95.6% specificity when compared to isolation of herpes simplex virus in tube cell culture. It was disappointing that the diagnostic advantage of shell vial assay seen with cytomegalovirus (68) and herpes simplex virus (69) was not realized for respiratory viruses in this study. The HEp-2 shell vial assay more efficiently isolated RSV than did conventional HEp-2 cell culture, however, immunofluorescent detection of RSV in DPS was the most efficient method. Although the diagnosis of RSV could be made by immunofluorescence alone, the HEp-2 shell vial assay could be a more accurate method of determining the efficiency of immunofluorescent methods than conventional cell culture, especially in situations when laboratory personnel are gaining experience with immunofluorescence. The A549 shell vial assay was equivalent to conventional cell culture for the isolation of adenovirus and RSV but was significantly less sensitive than cell culture for the isolation of influenza A and B and parainfluenza type 3 viruses. These results suggest that these cultured cells could not be used as a general purpose host system and corroborate similar findings (93,94). Perhaps the use of a different host cell (e.g. primary monkey kidney) would improve the utility of the shell vial assay for more rapid detection of

respiratory viruses from clinical specimens.

In conclusion, increased use of immunofluorescence for detecting viral antigens in clinical specimens and in cell cultures would allow many community hospital laboratories to offer virologic services. The BIFA procedure exhibited an acceptable level of sensitivity and specificity for the accurate and rapid diagnosis of respiratory virus infection. Immunofluorescence was judged to be convenient to use, cost competitive with other rapid diagnostic procedures and readily adaptable to laboratories equipped with a fluorescence microscope.

Since this study was initiated, there has been an increase in the number of requests for the laboratory diagnosis of respiratory virus disease. Rapid diagnosis of respiratory virus infections by immunofluorescence has gained general acceptance among the physician staff at the Quain and Ramstad Clinic as an important diagnostic tool.

SUMMARY

The incidence of respiratory viruses in Bismarck, North Dakota during consecutive respiratory disease years was determined by immunofluorescent detection of viral antigens in ciliated epithelial cells harvested from the respiratory tract and by conventional cell culture of throat swabs (TS), nasopharyngeal wash (NPW) and nasopharyngeal swab (NPS) specimens. These specimens were excellent tools for studying the epidemiology of respiratory viruses. Virus isolation rates of 7.7%, 33.2%, and 39.7% were achieved with TS, NPS and NPW specimens, respectively. Epidemics of respiratory syncytial virus (RSV) and influenza were observed during both years of the study. Months during which peak RSV activity occurred alternated from February of the first year to May of the second year. Parainfluenza virus and adenovirus activity was endemic in nature throughout the study period. Children less than 6 years of age and adults greater than 50 years of age had the highest viral morbidity. Of the specimens from infants less than 1 year old, approximately half were virus positive with RSV comprising 75.6% of the isolates. Influenza viruses were most commonly encountered in adolescents and adults.

Nasopharyngeal specimens were evaluated for the ability to harvest ciliated epithelial cells (CEC) from the upper respiratory tract. Specimens demonstrating $\geq$ 2 CEC per 400X field were judged to be acceptable for immunofluorescent assay. Overall, 8.7% of all nasopharyngeal specimens were unsuitable for immunofluorescent assay.

The probability of specimen rejection was type- and age-dependent. The rejection rate for NPW specimens was more than twice that for NPS specimens and the rejection rate for specimens from patients greater than 7 years of age was approximately 5 times greater than the rejection rate for specimens from patients less than 6 years of age.

Monoclonal antibodies from 2 commercial sources were used in immunofluorescent assays to detect virus antigens in respiratory CEC. When compared to cell culture, immunofluorescent assay was a sensitive and specific method for rapid diagnosis of respiratory virus infection. The Bartels indirect fluorescent antibody reagents had acceptable levels of sensitivity (overall 84.6%, range 33.3 - 100%) and specificity (overall 83.3%, range 87.3 - 100%). The Whittaker direct fluorescent antibody reagents had poor sensitivity (overall 50%, range 18.2 - 94.1%) and high specificity (overall 91.5%, range 93.8 - 100%). The highly efficient Bartels reagents for RSV and influenza A virus could be used as acceptable alternatives to culture for these viruses. The specificity of the influenza B, parainfluenza 3 and adenovirus reagents would result in a rapid presumptive diagnosis but the reagents are not sufficiently sensitive to replace culture.

Shell vial assays using HEp-2 and A549 cells offered no diagnostic advantage over immunofluorescent assay or conventional tube cell culture for the rapid identification of respiratory viruses.

Immunofluorescent assay using commercial monoclonal antibodies was a sensitive and specific method for rapid diagnosis of respiratory virus infections and has the potential to replace expensive and laborious conventional culture methods.

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