Peroxidase-Antiperoxidase Assay for Rapid Detection of Respiratory Syncytial Virus in Nasal Epithelial Specimens from Infants and Children

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A peroxidase-antiperoxidase (PAP) assay for the rapid detection of respiratory syncytial virus was compared with the indirect immunofluorescence method and with viral culture. Nasal epithelial specimens from 147 infants and children with acute respiratory infections were obtained and evaluated for the presence of respiratory syncytial virus antigens. Sensitivity, specificity, and accuracy by PAP were 91.7, 84.8, and 87.1%, respectively, and 87.0, 88.5, and 88.0%, respectively, by immunofluorescence compared with viral culture. The PAP assay was found to be as accurate as the indirect immunofluorescence method and more convenient to perform, since the color reaction and cell morphology were more easily observable by light microscopy. A new specimen collection method is reported; gentle scraping of the superficial nasal mucosa by the Rhino-probe method provided sufficient numbers of epithelial cells to perform multiple assays.

Respiratory syncytial virus (RSV) is the major cause of bronchiolitis in infants (3) and of nosocomial respiratory infections (11). Epidemics occur annually in large urban centers throughout the world. In the United States, thousands of infants are affected each year, many severely enough to require hospitalization. Infants with RSV bronchiolitis have been reported to have a greater than 50% chance of developing asthma later in childhood (5, 17, 18). The virus may also be the cause of apnea and unexpected deaths in this group of patients (12, 13). With the recent approval by the U.S. Food and Drug Administration of the antiviral drug ribavirin (Virazole; ICN Pharmaceuticals, Inc., Costa Mesa, Calif.) for the treatment of severe cases of RSV-induced bronchiolitis, methods for rapid detection of RSV could provide needed information for patient management and for controlling the spread of infections.

Direct and indirect immunofluorescence (IF) techniques have been applied to clinical specimens for the rapid detection of RSV (7, 9, 14–16). Limitations of these methods, such as the need for fluorescence microscopy and the evanescent nature of the staining, have inhibited the widespread use of these procedures in the clinical laboratory. Attempts to use immunoperoxidase methods, which use conventional light microscopy and provide long-lasting stained preparations, have resulted in contradictory findings (2, 6).

To date, the use of peroxidase-antiperoxidase (PAP) staining for rapid detection of RSV has not been evaluated. The purpose of this study was to compare the results of IF and PAP techniques for the rapid detection of RSV in clinical specimens from infants and children with acute respiratory disease with the results obtained by standard tissue culture isolation and identification methods. An evaluation of the specificity and sensitivity of the PAP method is reported.

Additionally, since the accuracy of these methods depends heavily on the quality of the nasal and pharyngeal specimens obtained, a new and improved method of specimen collection by use of the Rhino-probe scraper (Synbiotics Corp., San Diego, Calif.) is described.

MATERIALS AND METHODS

Patient population. The patients consisted of 142 infants and children with acute respiratory disease. They ranged from 2 weeks to 16 years of age. Most of these patients (78%) were sick enough to require hospital admission. They were hospitalized with the following diagnoses: acute respiratory distress, 26%; bronchiolitis-pneumonia, 44%; and croup and other diagnoses, 30%. Six patients from various other referral sources also took part in this study, performed from November 1984 to April 1985. The number of patients from each participating institution, which were all located in San Diego, Calif., were as follows: Children’s Hospital and Health Center, 72; University of California at San Diego (UCSD) Medical Center, 38; Mercy Hospital Medical Center, 16; and Sharp Rees-Stealy Clinic, 10. The hospitals and clinic are located within a 10-mile radius of the UCSD Medical Center, where the tests were performed. All patients had a nasopharyngeal swab specimen collected for viral culture and identification as part of their diagnostic evaluation. A second specimen was collected and processed in the manner described below for rapid detection of RSV antigen. All specimens were processed within 2 h of collection. A taxi courier system was instituted to bring the specimens to the laboratory from Monday through Saturday. Parental consent was obtained, and the research protocol was approved by the respective human experimentation committees.

Specimen collection. Two methods of collecting nasal and pharyngeal specimens were used. Before the specimens
were obtained, excess secretions were cleared with an aspirating bulb, and under direct visualization, a gentle scraping of the posterior portion of the inferior turbinate was performed by using a disposable plastic curette (Rhino-probe scraper) as reported earlier (4). The scraped cells were immediately placed in a plastic test tube (on ice) containing 1.5 ml of Hank's balanced salt solution without phenol red. The sample for viral culture was obtained from the same nostril with a Calgiswab Type 1 (Spectrum Diagnostics, Inc., Glenwood, Ill.) and placed in a vial containing veal infusion broth; the aluminum shaft was snapped, and the vial was capped tightly and placed on ice. Both specimens were then transported to the laboratories for analysis.

**Processing of specimens.** In the UCSD Viral Diagnostic Laboratory, standard viral culture and isolation techniques were used. Briefly, antibiotics were added to the specimen in veal infusion broth, which was then incubated at room temperature for 60 min. Aliquots (0.2 ml) of the specimen were planted into tubes of human embryonic kidney, HEp-2, and primary monkey (rhesus) kidney cells. Tubes showing cytopathic effect were identified by morphology and staining for RSV and herpes simplex virus and by neutralization for enterovirus and adenoviruses.

In the UCSD Rapid Diagnostic Laboratory, the scraped specimen in Hanks balanced salt solution was broken up into a fine cell suspension by use of a Pasteur pipette. The cell suspension was then centrifuged, and most of the supernatant was removed and discarded, leaving 0.2 to 0.3 ml of Hanks balanced salt solution in the test tube, in which the cells were resuspended. Specimens were then prepared for rapid viral diagnostic screening; a drop of the cell suspension was placed on each of several wells of microscope slides (Cel-Line Associates, Inc., Newfield, N.J.). The slides were air dried, fixed in anhydrous acetone, and air dried again before processing (or before storing at -70°C for later processing). This preparatory procedure took approximately 0.5 h.

**IF procedure.** A two-step IF method was used (14, 18), with bovine anti-RSV serum and bovine negative control, followed by fluorescein-conjugated antibovine serum (Burroughs Wellcome Co., Research Triangle Park, N.C.). The staining procedure took approximately 2 h.

**PAP procedure.** Briefly, guinea pig anti-RSV serum (Flow Laboratories, Inc., McLean, Va.) was adsorbed with human epithelial cells to remove anti-human antibodies; a previously determined dilution of the antiserum was applied to the specimen, and the slides were incubated for 20 min at room temperature. After the reagent was removed by vacuum, the specimen was rinsed in Trit-saline buffer and soaked for 5 min in Trit-saline. Excess buffer was again removed by vacuum, while the specimen was allowed to remain moist. Goat anti-guinea pig serum (Antibodies Inc., Davis, Calif.) was then applied, followed by similar incubation and rinsing procedures. In step 3, the PAP reagent (Jackson Immunoresearch Laboratories, Avondale, Pa.) was applied and incubated as before. After the specimen was rinsed and soaked, the substrate (aminethylcarbazole) was applied. The antigen-antibody complex was visualized under light microscopy by the presence of a red or reddish-brown coloration in the cytoplasm of respiratory epithelial cells. The staining procedure took approximately 2 h.

**RESULTS**

The number of patients from each participating institution and the number and type of specimens obtained are shown in Table 1. The majority of the specimens (97%) were obtained by the scraping technique, since preliminary studies demonstrated that this method yielded much larger numbers of respiratory epithelial cells when compared with the swab method; one gentle curettage may yield 50,000 to 100,000 epithelial cells. The scraping technique proved to be an efficient method of specimen collection; however, it remained to be proven that the cells were infected to the same extent as those collected more posteriorly with the nasopharyngeal swab method.

The mean ages of the patients in the RSV-positive and RSV-negative groups are compared in Table 2. Six additional patients from various sources were not included because their ages could not be determined. Patients from Sharp Rees-Staley Clinic (n = 10) were 12 or more months old and participated in this study because each had an upper respiratory infection at the time of the well-baby check-up at 12 months. Three of the ten babies (30%) were found to be positive for RSV by culture or PAP assay before July 1987. The mean age of RSV-positive patients was 5.7 ± 7.5 (standard deviation) months, in contrast to 11.7 ± 30.6 months for the RSV-negative infants and children. The majority of the patients (70%) were under 6 months old.

The average period from specimen collection to reporting results of viral culture was 13.1 ± 6.9 days. In contrast, the results of 70% of the PAP assays were reported the same day that the specimen was received, 15% were reported within 1 day, and the remaining 15% were reported within 48 h.

The average number of days from the onset of clinical symptoms to the time of presentation of the patient at the UCSD Medical Center was 12.9 ± 7.3 days. This number represents the average of the time from the onset of symptoms to the time of presentation to one of the two UCSD medical centers, since some patients were seen at both facilities.

**TABLE 1.** Participating institution, number of patients, and type of specimen obtained

<table>
<thead>
<tr>
<th>Institution</th>
<th>No. of patients</th>
<th>No. of specimens collected by:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rhino-probe</td>
</tr>
<tr>
<td>CHHC</td>
<td>72</td>
<td>77</td>
</tr>
<tr>
<td>UCSD</td>
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<td>38</td>
</tr>
<tr>
<td>Mercy</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>S R-S</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

* CHHC, Children's Hospital and Health Center; UCSD, UCSD Medical Center; Mercy, Mercy Hospital Medical Center; S R-S, Sharp Rees-Staley Clinic.

**TABLE 2.** Mean ages of RSV-positive and RSV-negative patients

<table>
<thead>
<tr>
<th>Institution</th>
<th>No. of patients</th>
<th>Mean age of patients (mo)</th>
<th>RSV-positive patients</th>
<th>RSV-negative patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>Mean age</td>
<td>No.</td>
</tr>
<tr>
<td>CHHC</td>
<td>72</td>
<td>11</td>
<td>32</td>
<td>5</td>
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<td>UCSD</td>
<td>38</td>
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<td>19</td>
<td>6</td>
</tr>
<tr>
<td>Mercy</td>
<td>16</td>
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<td>7</td>
<td>6</td>
</tr>
<tr>
<td>S R-S</td>
<td>10</td>
<td>20</td>
<td>3</td>
<td>13</td>
</tr>
</tbody>
</table>

* For institution abbreviations, see footnote to Table 1.
symptoms to the collection of specimens yielding positive cultures and positive results by the PAP assay was 6.1 ± 5.9 for 37 specimens. The average number of days for specimens yielding negative cultures and positive PAP assay results was 8.0 ± 6.9.

IF assay for RSV detection was performed in 142 of 147 specimens. In five specimens, the quantity of sample was only sufficient to perform the PAP assay. Results for prevalence, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of the test are given in Table 3, which is a fourfold table demonstrating the blind comparison of the IF method with viral culture. A total of 11 specimens were found to be positive by the IF method but negative by culture.

The results of 147 PAP assays compared with the results of viral culture are shown in Table 4; results for the same test characteristics (see above) are also reported. In the comparison, 15 specimens were found to be positive by PAP assay but negative by viral culture.

A summary comparing the three methods is shown in Table 5. The prevalence of RSV-positive specimens was 43%. Of the 15 specimens positive by the PAP assay, 8 were positive by the IF method; therefore, 7 specimens of 142 were RSV-positive by the PAP assay and not by culture or by the IF method.

DISCUSSION

The purpose of the present study was twofold: (i) to evaluate the PAP method for the rapid detection of RSV viral antigen in nasal epithelial cells and (ii) to determine the efficacy of the Rhino-probe nasal scraper as a method of collecting sufficient numbers of infected cells for rapid diagnosis. The evaluation was conducted by comparing the results of the PAP assay with those obtained by a well-established IF method and by viral culture.

Our interest in evaluating the PAP method is based on the theoretical advantages it offers over direct immunofluorescence and IF methods and over immunoperoxidase techniques used in the rapid diagnosis of RSV (19). The advantages of the PAP technique, such as increased sensitivity, the use of light microscopy, and the availability of a permanently stained preparation, as well as its disadvantages, such as the presence of nonspecific reaction due to endogenous peroxidase, have been discussed earlier (1).

Immunoperoxidase assays for the rapid detection of RSV have been used with conflicting results by two groups of investigators. In 1978, Gardner and co-workers, in a study sponsored by the World Health Organization, compared an IF antibody technique with an immunoperoxidase method for RSV rapid diagnosis (6). The results of the immunoperoxidase method were unsatisfactory mainly because of endogenous peroxidase, and measures to remove the endogenous peroxidase were unsuccessful because the process also destroyed the RSV viral antigen. These investigators concluded that the immunoperoxidase technique should not be used in clinical specimens until these problems had been solved.

In 1983, Cevenini and co-workers compared the results of an IF method with those obtained by an indirect immunoperoxidase method and by viral culture for the detection of RSV in nasopharyngeal secretions (2). The agreement among virus isolation and the indirect immunoperoxidase and IF methods was 89%. Endogenous peroxidase was not a problem in this study. The discrepancies between their findings and those reported by Gardner and co-workers were attributed to differences in the reagents used, especially with respect to peroxidase conjugate and substrate.

Our study differs from previous work in three major areas. (i) A PAP reagent was used for detection of the anti-RSV–RSV viral antigen immune complex. (ii) An efficient method for obtaining large numbers of nasal epithelial cells was used. (iii) All specimens were processed within a relatively short time after they were obtained, and they were handled under optimal experimental conditions.

The PAP reagent consists of an immune complex of the enzyme peroxidase with antiperoxidase antibodies, derived in the same species of animal as the primary antibody. The PAP reagent binds to the linking antibody, which is already attached to the primary antibody; the quantity of enzyme bound immunologically to the antibody is much greater than the number of antigen sites, and this has the effect of enhancing the color reaction and increasing the sensitivity of the assay. Other methods for labeling antibodies and amplifying the reaction even more, such as with the avidin–biotin technique (10), hold promise and are being evaluated.

Previous methods of specimen collection for rapid viral diagnosis have included swabbing the nasopharynx vigorously, aspirating nasopharyngeal secretions, and washing

### Table 3. Blind comparison between results of IF assay and viral culture

<table>
<thead>
<tr>
<th>IF assay result</th>
<th>No. of viral culture results</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>40</td>
<td>11</td>
</tr>
<tr>
<td>-</td>
<td>6</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>46</td>
<td>96</td>
</tr>
</tbody>
</table>

* The following IF assay characteristics, compared with those of viral culture, were calculated: prevalence, 88.5%; sensitivity, 87.0%; specificity, 88.5%; positive predictive value, 78.4%; negative predictive value, 89.4%; and accuracy, 88.0%.

### Table 5. Comparison of results of IF and PAP assays with results of viral culture

<table>
<thead>
<tr>
<th>Assay result</th>
<th>No. of viral culture results</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+, PAP+</td>
<td>40</td>
<td>8</td>
</tr>
<tr>
<td>-, PAP-</td>
<td>3</td>
<td>77</td>
</tr>
<tr>
<td>+, PAP-</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>-, PAP+</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>(ND*), PAP+</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>(ND), PAP-</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>47</td>
<td>100</td>
</tr>
</tbody>
</table>

* ND, Not done.
the nasopharynx with transport medium and then aspirating the wash (8). These techniques frequently provide specimens with inadequate numbers of cells, as well as much mucus and extraneous material requiring several washes to obtain a clean cell suspension.

The use of the Rhino-probe scraper eliminated the problem of having a scant number of cells for analysis and reduced the amount of interference with the assay procedure. Several slides with multiple spots of cell suspension can be prepared for rapid multiple-specimen screening. This method differs from previous ones in that nasopharyngeal secretions are removed by suctioning and are discarded and only a relatively clean superficial epithelial layer is sampled by gentle scraping. The procedure is quick and only mildly irritating, and no bleeding should occur when all safety precautions are taken. The Rhino-probe has been useful as an aid in the diagnosis of allergic or infectious rhinitis in infants (4) and is now routinely used in our institutions for this purpose and for rapid viral diagnosis.

Our experience in developing and using this new method for rapid detection of RSV is very similar to that of other investigators. Several months were devoted to the following tasks: finding good quality reagents, determining the appropriate dilutions, adsorbing interfering antibodies, acquiring sufficient technical experience, and maintaining reliable quality control measures.

In conclusion, we found the PAP assay as accurate as the IF test but more convenient to perform, since the color reaction and cell morphology were easily observed by light microscopy. Interference due to endogenous peroxidase was not a significant problem because respiratory epithelial cells were easily differentiated from polymorphonuclear leukocytes, which contain variable amounts of peroxidase. Elimination of endogenous peroxidase was found to be unnecessary. In addition, we found the Rhino-probe scraper useful in obtaining large quantities of respiratory epithelial cells for performing multiple assays for detecting RSV (and potentially other viral respiratory pathogens). The importance of rapidly diagnosing RSV infection in infants has reached a new level of significance with the recent development and approval by the U.S. Food and Drug Administration of antiviral therapy with ribavirin. The PAP method using Rhino-probe-collected epithelial cells is rapid, accurate, and dependable in establishing a diagnosis of RSV infection in infants with acute respiratory disease.

ACKNOWLEDGMENTS

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LITERATURE CITED