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Comparison of Nasopharyngeal Aspirate and Nasopharyngeal Swab Specimens for Respiratory Syncytial Virus Diagnosis by Cell Culture, Indirect Immunofluorescence Assay, and Enzyme-Linked Immunosorbent Assay

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Paired nasopharyngeal aspirate (NPA) and nasopharyngeal swab (NPS) specimens obtained from each of 32 hospitalized infants with X-ray-confirmed pneumonia (91%) or bronchiolitis were tested for respiratory syncytial virus (RSV) infection by virus culture, the indirect immunofluorescent-antibody (IFA) technique, enzyme-linked immunosorbent assay (ELISA; Ortho Diagnostic Systems, Inc.), and spot hybridization with a human genomic probe to quantitate cellular DNA. RSV was isolated in cell cultures from 72% (23 of 32) of patients by using NPA specimens compared with 47% (15 of 32) by using NPS specimens. With tissue culture positivity as the reference test, the sensitivities of the ELISA on NPA and NPS specimens were found to be 69% (16 of 23) and 61% (14 of 23), respectively, with a specificity and a positive predictive value from both sites of 100%. The sensitivities of the IFA technique compared with the cell culture on NPA and NPS specimens were 61% (14 of 23) and 52% (12 of 23) with specificities of 89 and 78% and positive predictive values of 96 and 92%, respectively. Despite the recovery of significantly more cells (as shown by detection of more cellular DNA by using NPA specimens), virus was detected by the IFA technique or ELISA at similar frequencies in paired specimens. However, virus was recovered more often from NPA than NPS specimens by cell culture, and ELISA optical density readings and the number of RSV-positive fluorescing cells were greater for NPA specimens. NPA specimen collection was less traumatic for the patient, was an easier procedure for the physician to perform, and provided a superior laboratory specimen for RSV diagnosis than the NPS technique.

Respiratory syncytial virus (RSV) is the most important respiratory pathogen of infants and young children, causing a yearly winter outbreak of lower respiratory tract infection (8). The presence of the virus in the community is signalled by a sharp increase in the incidence of bronchiolitis and bronchopneumonia, which frequently leads to the hospitalization of the patient. The development of ribavirin as a useful therapeutic agent has led to interest in implementing rapid methods for laboratory diagnosis of RSV infection (10, 25).

Rapid diagnosis of RSV has been carried out in several laboratories by using indirect immunofluorescent-antibody (IFA) staining of epithelial cells (2, 4, 6, 9, 13, 14, 17, 23, 26) and the enzyme-linked immunosorbent assay (ELISA) (3, 9, 12, 13, 16, 22, 24) for direct detection of RSV antigens in clinical specimens. An important component of the success of these techniques is the source of specimens and the time of sampling the specimen during the course of the infection. However, few studies have evaluated either the above rapid diagnostic methods relative to the use of culture or the specimen sample source. The objectives of this study were (i) to compare the results of nasopharyngeal aspiration (NPA) and nasopharyngeal swab (NPS) sampling specimens for RSV detection by using tissue culture, IFA staining, and ELISA; and (ii) to compare the cell yield of these sampling techniques as measured by cellular nucleic acid hybridization.

MATERIALS AND METHODS

Patients. From January to April 1986, paired NPS and NPA specimens were obtained from 32 infants admitted to the Children's Hospital with X-ray-confirmed pneumonia or bronchiolitis or both. Three infants had bronchiolitis alone. Infants ranged in age from 1 to 17 months (median, 4 months). Infants had been symptomatic for 0 to 14 days (median, 4 days) before admission to hospital. The time of sampling ranged from 2 to 16 days (median, 6 days) after the onset of symptoms.

Collection of specimens. The NPS sample was obtained first by the following procedure. A calcium alginate swab was introduced into the nose and gently pushed back toward the nasopharynx until the tip had passed the measured distance from the anterior naris to the ear or resistance was felt. The swab was rotated, removed, and placed immediately into 2 ml of the viral transport medium (tryptose phosphate broth containing 0.5% gelatin and antibiotics). The NPA specimen was obtained from the other naris. A no. 6 or no. 8 French catheter with a specimen trap was used for aspirating the mucous and cells. The catheter was removed, and 2 ml of transport medium was suctioned through the

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catheter into the trap, thereby washing any of the specimen remaining in the catheter into the trap. The trap was disconnected and sealed. Both NPS and NPA specimens were identified by number only and transported immediately at room temperature within 30 min to the Cadham Provincial Laboratory.

NPS and NPA specimens were processed identically. Each specimen was vortexed. A 0.5-ml portion was removed and stored at −20°C until ELISA testing. The remaining specimen was centrifuged at 1,500 rpm (500 × *g*) for 10 min. The supernatant was removed, treated with an equal volume of antibiotics (gentamicin, 100 U/ml; penicillin, 25 μg/ml; mycostatin, 25 U/ml), and used for inoculation of the cell culture. The sample cell pellet was washed three times with phosphate-buffered saline and suspended in 250 μl of phosphate-buffered saline containing 1% bovine serum albumin. A 25-μl portion of the suspended cell pellet was saved for spot hybridization. The cell density was adjusted for heavy suspension, and the cells were spotted on a slide, dried, fixed in cold acetone for 10 min, and stored at −20°C until tested by the IFA technique.

Virus isolation. (i) Cell culture. Antibiotic-treated specimens (0.2 ml each) were inoculated into tubes that each contained a monolayer of human epidermoid carcinoma cells (HEp-2), primary rhesus monkey kidney cells (RhMk), primary rabbit kidney cells (Rk), or human foreskin fibroblast cells (Huff). The cultures were incubated at 37°C in stationary racks and examined every other day for the characteristic cytopathic effect of RSV as well as for other viral agents. A repass on HEp-2 cultures was done on day 6. Cultures were examined for an additional 10 days. To detect the presence of myxo- and paramyxoviruses, a hemadsorption assay was carried out on primary rhesus monkey cell cultures on day 10. Huff and RhMk cultures were maintained for 10 days. The presence of RSV in HEp-2 cultures was determined by the typical cytopathic effect. Five RSV isolates, which were detected by cytopathic effect when the indirect immunofluorescent-antibody test or ELISA was negative, were confirmed by the IFA technique on cell culture.

(ii) ELISA. For direct detection of RSV antigens, ELISA was performed as specified in the instructions provided by the manufacturer (Ortho Diagnostic Systems, Inc., Raritan, N.J.) with 100 μl of each specimen and 10 μl of the sample treatment medium. The ELISA involved the use of the double-antibody sandwich technique, in which the capture antibody was a mixture of two monoclonal antibodies (one to RSV nucleoprotein antigen and the other to capsid antigen) coated on a microtiter well and peroxidase-conjugated anti-RSV serum was used as a detector antibody.

To determine the low and high cutoff points, absorbance values of 0.15 and 0.2 were added to the mean absorbance value of the two negative medium controls. A specimen was considered to be positive if the absorbance value was higher than the value of both cutoff points. The reading was considered equivocal if the readings fell between the high and low cutoff value. The test was repeated for specimens with equivocal results.

The specificity of the Ortho ELISA of RSV was checked with a panel of heterologous viral antigens including those for adenovirus type 2, herpes simplex virus types 1 and 2, echovirus types 6 and 9, poliovirus type 1, parainfluenza virus types 1 and 3, and influenza virus A/Philippines and B/USSR. A HEp-2 culture-grown RSV suspension with a 50% tissue culture infective dose of $10^{3.5}$/0.1 ml was twofold serially diluted in Hanks balanced salt solution with duplicate samples to determine the endpoint sensitivity of the ELISA.

IFA technique. For direct detection of RSV antigens, smears prepared from clinical specimens were examined by the standard IFA technique (4). Standardized bovine anti-RSV serum, negative control serum, and fluorescein-conjugated anti-bovine immunoglobin (Wellcome Diagnostics, Dartford, England) were used. Specimens were considered adequate if more than eight nasopharyngeal epithelial cells were present at ×10 magnification and were considered positive for RSV if the smear showed greater than 2+ particulate green cytoplasmic fluorescence in one or more nasopharyngeal epithelial cells.

Probe preparation. A 250-ng quantity of a 300-base-pair *Alu*I restriction fragment of human repetitive DNA (21) was radiolabeled (20) to a specific activity of 10^8 cpm/μg by using a nick-translation kit (Amersham Co., Oakville, Ontario, Canada) and [α-^{32}P]dCTP (3,000 Ci/mmol; Dupont Canada Inc. [NEN Products], Lachine, Quebec, Canada).

Hybridization procedure. NPA and NPS specimens (20 μl each) collected from 32 patients were spotted on a nylon hybridization membrane (Magna; Fisher Scientific Co., Ltd., Winnipeg, Manitoba, Canada). Colonies of *Haemophilus influenzae* and *Streptococcus mutans*, normal flora of the oral cavity, were grown overnight on a second membrane and used to assess the specificity of the probe for human DNA. A third membrane was spotted with serial 10-fold dilutions of HeLa cells in a range from 10^7 to 10^2 cells. This membrane was used to quantitate the number of cells in each of the clinical specimens. The material on the membranes was denatured by soaking the membranes in a solution of 0.5 M NaOH–1.5 M NaCl for 10 min and then neutralizing them in a solution of 0.5 M Tris (pH 7.4)–2.5 M NaCl. Material was fixed on the membranes by baking for 4 h at 68°C. The membranes were prehybridized for 3 h at 65°C in a solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.5% sodium dodecyl sulfate, 5× Denhardt solution (50× Denhardt solution contains 1% [wt/vol] Ficoll 400 [Pharmacia Fine Chemicals, Piscataway, N.J.], polyvinylpyrrolidone, and bovine serum albumin fraction V), and 100 μg of denatured sheared salmon sperm DNA per ml. The nick-translated probe, denatured by boiling for 2 min, was added to a final concentration of 10 ng/ml. Hybridization was carried out for 16 h at 65°C. Nonspecifically hybridized probe was removed by washing the membrane for 30 min at 65°C in 2× SSC–0.1% sodium dodecyl sulfate and then for an additional 30 min in 0.1% SSC–0.1% sodium dodecyl sulfate. Autoradiography was carried out for 24 h with X-OMAT XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.).

Determination of cell number. The autoradiograph of the hybridized HeLa cells was scanned with a model 620 densitometer (Bio-Rad Laboratories, Richmond, Calif.). Each radiographic image was scanned over five serial sections of 2 mm in width. After each section was scanned, an integrated area for each specimen was calculated by averaging the values obtained from the five serial scans. The integrated area under the trace peaks was used to construct a standard curve relating area to cell number. The autoradiograph of the membrane spotted with the clinical specimens was scanned under identical conditions. An estimate of the number of cells in each specimen was obtained by reference to the standard curve described above.

Statistical analysis. As the NPA and NPS collected from each patient were paired specimens, a one-tailed paired *t* test was used to compare integrated areas calculated from the

TABLE 1. Comparison of results of diagnostic techniques in the detection of RSV-positive NPA specimens

No. of RSV-positive specimens by:		
Cell culture	IFA staining[a]	ELISA[b]
13	13	13
3	0	0
1	1	0
2	0	2
2	0	E[c]
1	NSQ[d]	1
1	NSQ	0

[a] Sensitivity of IFA technique = 100 × 14/21 = 67%.
[b] Sensitivity of ELISA = 100 × 16/21 = 76%.
[c] E, Equivocal.
[d] NSQ, Not sufficient quantity of cells for IFA testing.

densitometer traces of the autoradiograph. The null hypothesis was that there would be no difference in the number of cells collected by the two techniques; the alternate hypothesis was that the NPA technique collected more cells than the NPS technique.

RESULTS

Virus detection. A comparison of rapid diagnostic procedures with the standard of virus culture for the detection of RSV in NPA and NPS specimens is presented below and in Table 1. Of the 32 infants in the study, 23 (72%) were RSV positive by cell culture with HEp-2 cells. All 23 NPA samples (72% of the total 32) were positive by cell culture, but only 15 of the 32 NPS specimens (47%) were positive by cell culture. The difference in RSV isolation from the two sources of specimens was found to be significant by McNemar's test for paired proportions ($P < 0.05$) (5). For NPA and NPS specimens, an average of 9 and 11 days, respectively, was for required a final diagnosis by cell culture, with a range of 2 to 16 days overall.

Using tissue culture positivity as the reference standard, RSV could be detected by ELISA in 69% (16 of 23) of NPA and 61% (14 of 23) of NPS specimens. The test specificity was 100% on initial testing of NPA specimens by ELISA. Three specimens gave equivocal results; two of these were positive by tissue culture. After the test was repeated with these specimens, two remained equivocal (positive by tissue culture) and one was negative (negative also by cell culture). Two NPS specimens (negative by cell culture) gave equivocal results; one remained equivocal upon repeat testing. Figure 1 compares the absorbance value of NPS and NPA specimens, when both were positive for RSV antigen. The absorbance values for NPA specimens were statistically greater than for the NPS specimens, indicative of a higher virus titer in the NPA specimens.

The RSV ELISA did not show cross-reaction with other viruses. ELISA was found to be approximately 100-fold less sensitive than the cell culture endpoint (3.2×10^1 50% tissue culture infective doses per 0.1 ml versus $1 \times 10^{3.5}$ 50% tissue culture infective doses per 0.1 ml, respectively). These dilution studies also demonstrated that ELISA results were very susceptible to the dilution effect, with a sharp drop in absorbance values over as small as two- and fourfold dilutions (data not shown).

By including results from using both NPS and NPA specimens, we found that a total of 18 culture-confirmed specimens were IFA positive. With the NPA specimen only, 61% (14 of 23) of the culture-positive specimens were also positive by IFA. One specimen was positive by IFA but negative by tissue culture. Four NPA specimens were found to have insufficient cells for testing by IFA; two of these were positive by cell culture. With NPS specimens only, 52% (12 of 23) of the culture-positive specimens were positive by IFA. Two specimens were falsely positive. Three NPS specimens has insufficient cells for testing by IFA; two of these were positive by cell culture. Of the 18 culture-confirmed IFA-positive specimens, both NPA and NPS specimens were IFA positive in eight patients, the NPA specimen only was IFA positive in six patients, and the NPS specimen only was IFA positive in four patients.

In situ hybridization. The human repetitive DNA probe did not hybridize with herring sperm, *H. influenzae*, or *S. mutans* DNA (data not shown), indicating that autoradiographic signals were not due to nonspecific hybridization.

Estimation of cell number. As determined from the densitometer readings, significantly more cells were obtained by the NPA technique than by the NPS technique (t_{31df} [$P < 0.05$] = 2.06).

DISCUSSION

Specimens used most frequently for the detection of RSV from the respiratory tract have included throat swabs (17, 26), nasal swabs (1, 2, 11, 26), nasopharyngeal washes (2, 10, 14, 26), nasal and throat swabs (18), and nasopharyngeal aspirates (3, 4, 6, 7, 9, 12, 13, 16, 22, 24). Nasopharyngeal washes are not routinely performed in some institutions, perhaps because of possible patient discomfort if they are not well performed. Our observation was that infants tolerated sample collection by NPA much better than by use of nasopharyngeal swabs.

Few studies have directly compared virus yields from samples taken from infants by more than one sample collection method. McIntosh et al. took further samples from nine infants known to be excreting RSV and found the NPA yield greater than the NPS yield by ELISA (six versus one positive) and by culture (eight versus three positive) (16). Cradock-Watson et al. observed that NPA specimens were superior to throat swabs because they yielded greater numbers of cells for fluorescence examination, although no data were presented (4). Hall and Douglas documented that the

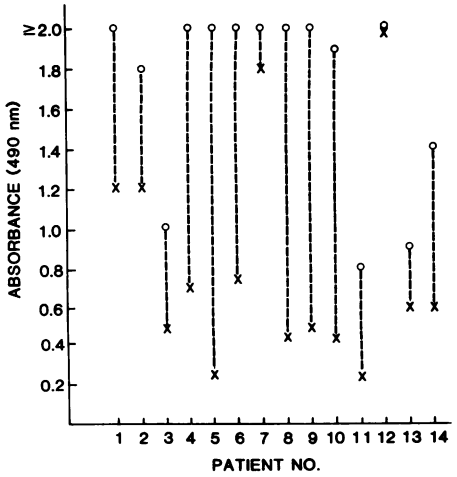

FIG. 1. Comparison of absorbance values for NPS and NPA specimens positive for RSV by ELISA. Each dashed line connects the NPS and NPA ELISA absorbance values from each child.

yield of RSV by culture from nasopharyngeal washes was statistically superior to that from NPS specimens. Additionally, they documented a superior virus titer and a shorter time to detection of RSV in cultures from nasopharyngeal wash specimens (10). Treuhaft et al. showed the superiority of nasopharyngeal wash specimens over both throat swab and NPS specimens (26). Thus, comparative studies point to the nasopharynx as the optimal RSV detection site.

From our study of 32 patients, NPA specimens were significantly better than NPS specimens on the basis of virus isolation results. Virus was detected more often by all techniques in NPA than in paired nasal swab specimens. The NPA technique also provided significantly more human cells than the NPS technique. We believe that when the nasopharyngeal wash technique is not an accepted sampling method, the NPA technique provides the optimum laboratory specimen for RSV diagnosis.

NPA specimens have been shown in one study to be better than NPS specimens for testing by ELISA (16). In our study, two additional infants were positive for RSV when NPA specimens were used for testing by ELISA, but the numbers of specimens tested were too small to detect a statistical difference in the ELISA results on the basis of the sampling site. In addition, the ELISA absorbance values of RSV-positive NPA specimens were significantly higher than the NPS specimen values, indicating a higher titer of virus at the NPA sampling site, supporting the previous study of McIntosh et al. (16). The reported sensitivity of RSV ELISA detection between laboratories has varied from 75 to 88% (3, 24). In our laboratory, the RSV ELISA sensitivity was only 69%. This variation in sensitivity may be due to differences in times of sampling, culture methods, or, possibly, virus strains. The ELISA may have decreased sensitivity if specimens are diluted (9), which did not occur in our study, or if washed-cell fractions are used (12). However, our results show that the Ortho ELISA was very specific, in agreement with other studies (3, 24). Further improvement may be possible with biotin-avidin conjugates for ELISA detection of RSV (12).

The sensitivity of the IFA procedure for RSV antigen detection was similar to that of ELISA in our study and those of others (9, 12, 13, 15, 16, 22, 24). For the IFA procedure, NPA specimens were better than NPS specimens owing to the greater number of cells collected by the former procedure. Although the IFA technique is rapid, it is less readily adapted as a routine procedure because of difficulties with specimen preparation and reader subjectivity. Unfortunately, apparent false-positive results may occur when specimens are collected at a later stage of the disease (15) or if transport and virus culture are suboptimal. However, the IFA technique will benefit greatly from the use of monoclonal antibodies, which have shown results comparable to polyclonal reagents (2).

In our laboratory experience, the rapid diagnosis of RSV infections by ELISA and the IFA procedure is best facilitated by use of NPA specimens, with virus culture as a backup technique if the above rapid test(s) are negative.

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