

## Practical Recommendations for the Detection of Pediatric Respiratory Syncytial Virus Infections

MARY W. TREUHAFT,\* JOLEEN M. SOUKUP, AND BRADLEY J. SULLIVAN

*Marshfield Medical Foundation and the Marshfield Clinic, Marshfield, Wisconsin 54449*

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**In our private clinic-hospital setting, respiratory syncytial virus (RSV) was isolated from infants more frequently and sooner from nasal washes (84%; 4.2 days) than from throat swabs (45%; 5.5 days) or nasopharyngeal swabs (39%; 5.7 days). Immunofluorescence of nasal wash cells identified 72% of the infants with virus isolations from nasal washes in less than one day. We therefore recommend the combination of isolation and immunofluorescence on nasal wash specimens for optimal detection of RSV-infected infants. Immunofluorescence of respiratory tract cells was also useful for monitoring the presence of RSV antigen in intubation secretions during ribavirin antiviral therapy. RSV infectivity was maintained in phosphate-buffered saline at room temperature for 6 h. Transport and inoculation of specimens in <6 h yielded RSV isolates from 50% of sampled infants during the two RSV seasons examined. For optimal RSV isolation, we recommend inoculation of HEP-2 tubes  $\leq 4$  days old. Replacing medium after 3 days as compared with 7 days did not increase recovery of RSV and provided little practical reduction in time to detection of cytopathology.**

To maximize detection of respiratory syncytial virus (RSV) pediatric respiratory infections in our private clinic-hospital setting, we examined a variety of parameters to enhance virus isolation. We also assessed the usefulness of rapid detection of viral antigen in respiratory tract cells by using commercial immunofluorescent reagents. Although bedside inoculation of cell culture tubes with nasal washes has been reported to give excellent RSV recovery (4), it is not practical in our medical center. Therefore, we first substantiated the recovery of RSV infectivity from the phosphate-buffered saline (PBS) used for nasal washes and then demonstrated recovery of infectious virus after transport of the specimens to the laboratory for inoculation. Because throat swabs and nasal swabs are often recommended for RSV isolation (5, 8, 9) and are easier to obtain than nasal washes, we compared all three specimens for virus isolation. Finally, we examined nasal washes, nasal swabs, and tracheal secretions for respiratory tract cells suitable for rapid detection of virus by immunofluorescence.

### MATERIALS AND METHODS

**Patients.** Specimens were obtained from hospitalized infants or those presented to the Marshfield Clinic with bronchitis or pneumonia of suspected viral etiology. This study was reviewed and approved by the local institutional review board, and permission was obtained from the parents of the patients. Parental permission was also obtained for emergency therapy with the investigational drug, ribavirin, for the one infant reported here.

**Cells and medium.** HEP-2 cells (CCL 23) obtained from the American Type Culture Collection, Rockville, Md., were maintained in minimum essential medium with modified Eagle salts (Flow Laboratories, McLean, Va.) supplemented with 5% fetal bovine serum, 2 mM glutamine (M. A. Bioproducts, Walkersville, Md.), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; Research Organics, Inc., Cleveland, Ohio), 2  $\mu$ g of amphotericin B (Fungizone; E. R. Squibb & Sons, Princeton, N.J.) per ml, 100 U of penicillin G per ml, and 100  $\mu$ g of streptomycin (Eli

Lilly & Co., Indianapolis, Ind.) per ml (MEM-5% FBS). Plastic tissue culture tubes were set with  $1.5 \times 10^5$  cells per tube, incubated overnight at 37°C, and maintained at 32°C until used for RSV isolation.

**Specimens.** During the 1983 RSV epidemic season, throat swab and nasal wash specimens were obtained from all patients. During the 1984 season, nasopharyngeal swabs were also obtained. Specimens were collected in the following order to prevent multiple sampling from the same site: throat swab, nasopharyngeal swab from one nostril, and nasal wash from the other nostril.

Throat swab samples were obtained with viral Cultures (Marion Scientific, Kansas City, Mo). Swabs were rotated and thoroughly extracted in 1.5 ml of MEM-5% FBS, which was used for virus isolation. Nasopharyngeal swab samples were obtained with Calgiswabs (Inolex, Glenwood, Ill.) immersed in 2 ml of medium and vortexed for 10 s. After centrifugation at  $1,500 \times g$  for 10 min, the supernatant fluid was used for virus isolation, and the cell pellet was used for immunofluorescence. Nasal washes were obtained with 5 ml of PBS by the procedure of Hall and Douglas (4). Nasal washes and tracheal secretions were vortexed for 10 s to release cells from mucus and to release cell-associated virus from infected cells. After centrifugation, the supernatant fluid was used for virus isolation, and the cell pellet was washed two or three times in PBS to remove residual mucus before being used for immunofluorescence. Tracheal secretions were obtained from hospitalized infants requiring intubation for ventilatory assistance. Specimens were transported to the laboratory at room temperature and were inoculated within 2 h of receipt.

**RSV isolation.** Duplicate tubes of HEP-2 indicator cells ( $\leq 4$  days old) were inoculated with 0.2 ml of specimen and adsorbed for 2 h at 35°C. The inoculum was then removed, and 1 ml of medium was added. Tubes were incubated at 35°C and observed daily for characteristic RSV syncytial cytopathology. Identity of the virus was confirmed by immunofluorescent staining of cells from tubes exhibiting  $\geq 50\%$  RSV cytopathology.

**Immunofluorescence.** RSV antigen was detected in respiratory tract cells and in cells from virus isolation tubes by

\* Corresponding author.

TABLE 1. Stability of RSV in PBS<sup>a</sup>

Medium	% of initial titer after:			
	6 h		24 h	
	25°C	4°C	25°C	4°C
MEM-2% FBS	60	60	8	19
PBS	70	60	20	13

<sup>a</sup> A pool of RSV (laboratory-passaged strain) containing  $2 \times 10^7$  PFU/ml was diluted 1:100 in the medium indicated and placed in tubes (1 ml per tube); tubes were incubated at either 25 or 4°C (on ice). At 0, 6, and 24 h, the mean surviving virus for each diluent-temperature combination was determined by plaque assay (11) of three replicate tubes in quadruplicate ( $n = 12$ ). The coefficient of variation for the estimates ranged from 15 to 26%.

using commercial indirect immunofluorescent reagents (Burlington-Wellcome Corp., Research Triangle Park, N.C.) and was performed as recommended by the manufacturers.

RESULTS

**Stability of RSV in PBS.** The stability of RSV in PBS over the time required for transport of nasal wash specimens to the laboratory was examined and compared with that in a conventional transport medium, at both room temperature and on ice. As determined by plaque assay (11) of a passaged laboratory strain, 60 to 70% of initial infectivity was retained over a 6-h period, and RSV was as stable in PBS as in MEM-2% FBS (Table 1). By 24 h, however, 80% of the infectivity was lost in both transport media. Holding specimens on ice showed no advantage over holding at room temperature for either time period. Although these results do not reproduce field conditions, they do suggest that RSV can be efficiently isolated from specimens transported at room temperature to the laboratory and inoculated within 6 h. With prompt transport and inoculation, 50% of the nasal wash specimens submitted yielded infectious RSV in the 1983 epidemic season (February through April). In the 1984 season (January through March), 51% of the specimens submitted yielded infectious RSV.

**Age of HEp-2 culture tubes.** We observed that laboratory strains of RSV replicated optimally and produced cytopathology most rapidly when the HEp-2 host cell monolayers were barely confluent (70 to 80%) at the time of inoculation (data not shown). Many diagnostic laboratories prepare HEp-2 isolation tubes only weekly, and even when they are held at 32°C, there is sufficient cell division by 6 to

TABLE 2. Effect of age of HEp-2 culture tubes on RSV isolation<sup>a</sup>

Inoculation size (PFU)	Age of culture tubes (days)	No. of tubes positive/no. of tubes examined				
		Day 2	Day 3	Day 4	Day 5	Day 6
3	≤4	—	—	4/8	8/8	8/8
	>4	—	—	—	4/6	6/6
10	≤4	—	4/8	7/8	8/8	8/8
	>4	—	—	4/6	6/6	6/6
100	≤4	—	7/7	7/7	7/7	7/7
	>4	—	1/6	6/6	6/6	6/6

<sup>a</sup> HEp-2 cells were set at  $1.5 \times 10^5$  cells per tube and maintained at 32°C. At days 1, 2, 3, 4, 5, 6, and 7 after preparation, tubes were infected with 3, 10, or 100 PFU of RSV and incubated at 35°C. Tubes were scored as positive when they exhibited typical RSV cytopathology. —, No cytopathic effect observed in any specimens examined.

TABLE 3. Comparison of specimens and techniques for detection of RSV infection

Specimen, technique	No. of detections/ no. of specimens (%)	Time (days) to detection	
		Mean	Range
Tracheal secretions			
Isolation	2/3 (67)	4.0	
Immunofluorescence	2/3 (67)	<1.0	
Nasal wash <sup>a</sup>			
Isolation	26/31 (84)	4.2 <sup>b</sup>	2-10
Immunofluorescence	19/31 (61)	<1.0	
Throat swab <sup>a</sup> (isolation)	14/31 (45)	5.5 <sup>b</sup>	3-11
Nasopharyngeal swab <sup>a</sup>			
Isolation	9/23 (39)	5.7 <sup>b</sup>	2-12
Immunofluorescence	2/7 (29)	<1.0	

<sup>a</sup> Paired specimens. In the 1983 RSV season, paired nasal wash and throat swab specimens were obtained from eight RSV-infected infants. In the 1984 RSV season, paired nasal wash, throat swabs, and nasopharyngeal swabs were obtained from 23 RSV-infected infants.

<sup>b</sup> As calculated by using the paired Student's *t* statistic for patients from whom RSV was isolated from both specimens, virus was detected sooner in isolation tubes inoculated with nasal washes than in tubes inoculated with throat swabs ( $P < 0.05$ ;  $n = 11$ ) and sooner with nasal washes than with nasopharyngeal swabs ( $P < 0.05$ ;  $n = 8$ ).

7 days to produce densely crowded monolayers which may be less than optimal for efficient RSV isolation. To evaluate whether the age of isolation tubes and concomitant cell density does indeed influence ability to isolate RSV, we inoculated tubes with a range of RSV infectious units (3, 10, or 100 PFU) over 1 to 7 days after tube preparation. The first day on which tubes exhibited RSV cytopathology was recorded (Table 2). With a high, as well as a low, viral inoculum, tubes inoculated 1 to 4 days after preparation regularly exhibited cytopathology sooner than did tubes inoculated after 5 days or more. Thus, preparing HEp-2 indicator tubes twice weekly so that tubes 4 days old or less are continually available for inoculation should result in more rapid RSV isolation.

**Comparison of specimens for RSV isolation.** Recovery of infectious RSV from infected infants was compared for three types of specimens during the 1984 epidemic season and for two types of specimens during the 1983 season. Infants were considered to be infected with RSV if virus was isolated from any specimen or if antigen was detected by immunofluorescence of respiratory tract cells.

RSV was isolated more frequently from nasal washes (84%) than from either throat swabs (45%) or nasopharyngeal swabs (39%) (Table 3). Additionally, virus was detected by viral culture significantly earlier from nasal washes than from the other two specimens, probably because the nasal washes contained more virus. Of the five infants with negative isolation results on nasal wash specimens, one exhibited RSV antigen in nasal wash cells, although virus was not recovered from any site. For the other isolation-negative infants, virus was isolated from only one site (three from throat swabs and one from the nasopharyngeal swab) and was detected relatively late (days 8, 5, 7, and 6) and in only one of the duplicate tubes inoculated. These observations suggest that these five infants with negative isolation results on nasal washes were all shedding low amounts of virus and that recovery of virus may have depended more on chance than on type of specimen.

**Effect of replacing medium on RSV isolation.** When laboratory strains of RSV are passed at low multiplicity of infection, an occasional culture showing little cytopathology by 3 to 4 days may be seen to exhibit extensive cytopathology within 24 h after replacement of the culture fluid with fresh medium. Therefore, we evaluated whether replacing the medium would increase RSV isolation from clinical specimens or reduce the time to detection of cytopathology. Duplicate isolation tubes were inoculated with clinical specimens, culture fluid was passed to new tubes, and medium was replaced at either day 3 or day 7. Replacing the medium at day 3 as compared with day 7 did not increase isolation for any of the specimen types (data not shown). In addition, no practical or statistically significant decrease in the time to detection for any specimen type was observed (data not shown: for nasal washes  $n = 24$ ; for throat swabs,  $n = 13$ ; for nasopharyngeal swabs,  $n = 8$ ; and for tracheal secretions,  $n = 2$ ).

**Usefulness of immunofluorescence.** Immunofluorescence of nasal wash cells was effective in identifying 61% of our RSV-infected infants within 1 day (Table 3). When only isolation-positive nasal washes with adequate numbers of cells are considered, the identification rate increases to 72%. Only one nasal wash (4%) was positive by immunofluorescence but failed to yield infectious virus. Of a total of 63 nasal washes submitted, only 7 (11%) had insufficient cells for immunofluorescence. In contrast to nasal washes, nasopharyngeal swabs were relatively unsatisfactory for identification of infection by immunofluorescence (Table 3).

Immunofluorescence was also successful in detecting RSV antigen in cells from tracheal secretions (Table 3). This approach was particularly useful in following the presence of RSV in one infant treated with ribavirin antiviral therapy in which tracheal secretions never yielded infectious virus (probably because they were obtained by elution of tubing with distilled water). Viral antigen was detected in cells from tracheal secretions on days 1 through 6 and day 8 during an 11-day course of ribavirin therapy.

## DISCUSSION

Our isolation of RSV from specimens submitted (50% for 1981 and 51% for 1983) without bedside inoculation or transport on ice suggests that prompt transport to the laboratory and inoculation are adequate for good recovery of RSV. After completion of this study, Bromberg et al. (2) reported that transport of nasopharyngeal aspirates at 4°C to the laboratory for inoculation yielded an RSV isolation rate very similar to that obtained by immediate inoculation at the bedside. Whether transport of nasal washes at 4°C can improve RSV recovery beyond what we observed here for transport at ambient temperature will require a prospective comparative study.

Culture tubes  $\leq 4$  days old exhibited cytopathology sooner upon inoculation with a laboratory strain of RSV than did older tubes. Therefore, for efficient RSV isolation, we recommend preparing HEp-2 isolation tubes twice weekly and holding them at 32°C until inoculation so that low-density monolayers are continuously available during RSV epidemic periods.

This study confirms previous reports that nasal washes are superior to either throat swabs (12) or nasopharyngeal swabs (4) for virus isolation and our isolation rates for throat swabs and nasopharyngeal swabs were similar to those previously reported. The combination of virus isolation and immunofluorescence on nasal wash specimens permitted detec-

tion of infection rate in 84% of infants examined, with 61% of the infants identified within 1 day. Although sampling multiple sites (nasal washes, throat swabs, and nasopharyngeal swabs) increases the chance of recovery from infants shedding low levels of virus, this is neither practical nor cost effective. Tracheal secretions, like nasal washes, were a good source of both infectious virus and respiratory tract cells for immunofluorescence, whereas nasopharyngeal swabs were not very useful for immunofluorescence. Replacing medium at day 3 rather than waiting until day 7 provided no practical benefit in terms of virus recovery or time to detection.

The significant advantage of immunofluorescence over isolation is timely information for the clinician. With the availability of effective antiviral therapy, this advantage becomes even more attractive. The rapid results of immunofluorescence also allowed us to follow the presence of RSV during antiviral therapy. Ability to monitor the presence of viral antigen should be very useful to the clinician in effectively using new antiviral therapies.

Our identification rate of RSV in nasal washes by immunofluorescence of 72% relative to virus isolation compares very favorably with previous reports of others using either nasal washes or nasal secretions in combination with polyclonal or monoclonal antibodies: 45% (12), 92% (3), 92% (10), 95% (7), 83% (1), and 89% (6).

Although immunofluorescence does require laboratory personnel experienced in interpretation, the characteristic cellular staining pattern allows identification with only a few infected cells on a slide. The other major requirement for successful identification by immunofluorescence is high-quality, specific reagents such as those currently available for RSV. Hopefully, commercial manufacturers will provide these for an expanded spectrum of infectious agents in the near future.

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