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SimulFluor Respiratory Screen for Rapid Detection of Multiple Respiratory Viruses in Clinical Specimens by Immunofluorescence Staining

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A new rapid direct immunofluorescence assay (DFA) respiratory screen reagent for detection of seven common respiratory viruses (respiratory syncytial virus [RSV], influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus) was compared with standard single or dual DFA reagents and culture. In total, 1,531 respiratory samples were adequate for testing with both SimulFluor Respiratory Screen (RS) reagent (Chemicon International, Temecula, Calif.) and single or dual DFA reagents. The RS DFA reagent detected 367 (98.4%) and single or dual DFA reagents detected 368 (98.7%) of 373 DFA-positive samples. In addition, the RS DFA reagent was equivalent to or better than culture for detection of all viruses except adenovirus. Only 15 of 799 (1.9%) RS-negative samples inoculated into cell cultures yielded respiratory virus isolates (one RSV, five influenza A virus, two influenza B virus, one parainfluenza virus, and six adenovirus). Sixty-six other virus isolates (13 rhinovirus, 24 cytomegalovirus, 28 herpes simplex virus type 1, and 1 enterovirus) were also recovered in culture. With cytospin preparation of slides, only 7.5% of samples submitted were deemed inadequate for DFA. The availability of a rapid DFA screening reagent for detection of multiple common respiratory viruses within 1 to 2 h of sample collection should be of great benefit in terms of patient management and infection control.

The importance of respiratory viruses as pathogens in children has long been recognized, and their impact in adults and in immunocompromised hosts has recently received greater recognition (14–16). Rapid diagnosis, while the patient is in the emergency room, is important to cohort patients on admission and implement proper infection control measures. Furthermore, new antiviral therapies which must be administered early to have a therapeutic impact and can be lifesaving in impaired hosts are becoming available (3).

A number of laboratory techniques can be used for the diagnosis of respiratory viruses, and they differ in sensitivity, cost, and time to results. Virus isolation in cell culture is sensitive and detects a broad spectrum of viruses; however, the time to results averages 6 days for respiratory syncytial virus (RSV), parainfluenza virus, and adenovirus and 2 days for influenza A and B viruses, and it can sometimes be as long as 14 days (5). Shell vial centrifugation cultures have been used to shorten the time to results to 1 to 5 days (2, 9, 11, 13). Providing a broad diagnosis by this approach requires incubation and staining of duplicate cultures of two different cell lines.

Recently, multiplex PCR has been reported as a rapid method for detection of multiple respiratory viruses with a sensitivity that may exceed that of culture (4). However, PCR assays take at least 6 to 8 h to complete and are generally performed no more than once a day; in smaller laboratories, they are done only once or twice a week. In addition, separate rooms and specialized equipment are needed, and reagents are expensive.

Rapid diagnostic methods such as membrane enzyme-linked immunosorbent assay provide results in 30 min and are simple to perform. Unfortunately, enzyme-linked immunosorbent assays are available for only influenza A virus and RSV and are often of suboptimal sensitivity (7).

Monoclonal antibodies for direct immunofluorescence assay (DFA) of cell smears for RSV, influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus are commercially available. Until now, detection of these viruses by DFA has required the preparation and examination of three to seven cell spots. A new respiratory screen reagent, SimulFluor Respiratory Screen (RS; Chemicon International, Temecula, Calif.), that permits direct DFA detection of all seven viruses in one cell spot is now available. This reagent utilizes a reddish-gold (rhodamine) label for RSV and an apple green (fluorescein) label for influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus. Therefore, if green-stained cells are visualized, a second slide must be stained to determine which of the last group of viruses is in the sample.

In this report, the performance of the SimulFluor RS reagent was compared with those of single and dual DFA reagents and/or culture of respiratory samples submitted to the Clinical Virology Laboratory of Yale New Haven Hospital from October 1998 through March 1999. To enhance readability and reduce the number of inadequate samples, all slides for use in DFA were prepared by cytocentrifugation (1, 6).

MATERIALS AND METHODS

Samples. A total of 1,673 samples, including nasopharyngeal (NP) aspirates (45%), NP swab specimens (47%), throat swab specimens (3%), bronchoalveolar lavage (BAL) fluids (3%), and assorted other specimen types (2%), were submitted to the Clinical Virology Laboratory at Yale New Haven Hospital from October 1998 through March 1999 for respiratory virus testing. Samples were tested by DFA for specific viruses and/or by culture depending on the virus(es) suspected and physician requests.

Slide preparation. Swabs in viral transport medium were vortexed, wrung out, and then discarded. To remove mucous from NP aspirates, 5 ml of phosphate-buffered saline (PBS) was added and specimens were pipetted up and down. Samples were then centrifuged at 700 × *g* for 5 min to pellet cells for DFA. The

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TABLE 1. Comparison of SimulFluor RS DFA with single and dual DFAs

Virus	Total no. of samples tested	No. (%) of positive samples			*P* value[a]
		Total	By RS (%)	By single or dual DFA (%)	
RSV	266	62	62 (100)	62 (100)	NA
Influenza A virus	1,431	238	232 (97.5)	236 (99.2)	0.157
Influenza B virus	1,431	38	38 (100)	38 (100)	NA
Parainfluenza virus types 1 to 3	287	22	22 (100)	19 (86.4)	0.083
Adenovirus	287	13	13 (100)	13 (100)	NA
Total	1,531	373	367 (98.4)	368 (98.7)	0.763

[a] Determined by McNemar's test. NA, not applicable.

cell pellets were resuspended in a small amount of PBS, and 200 μl of the suspension was applied per cell spot by cytocentrifugation (Cytospin 3; Shandon Inc., Pittsburgh, Pa.) at 800 rpm for 4 min. Slides were air dried and then fixed in cold acetone for 10 min. For the purpose of the study, two to three cytospin slides were prepared depending on the viruses suspected.

Respiratory screen DFA. Cell spots were stained with 40 μl of SimulFluor RS reagent (Chemicon International) for 15 min at 37°C. Following a 30-s wash in PBS, slides were mounted in glycerol, examined for the presence of fluorescein-labeled cells by the use of a fluorescein filter (for detection of influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus), and then reexamined for the presence of rhodamine-labeled cells by the use of a rhodamine filter (for detection of RSV). When fluorescein-positive cells were detected, a second and, occasionally, a third cell spot were stained to determine the infecting virus.

Single- or dual-reagent DFA. A total of 1,531 samples were also stained with single (RSV DFA kit; Bartels Inc., Issaquah, Wash.) and/or dual SimulFluor Influenza A/B and SimulFluor Parainfluenza 1,2,3/Adenovirus (Chemicon International) DFA reagents depending on the virus(es) suspected. The procedure was similar to that described above for RS, except that the Influenza A/B reagent slides were incubated with antibody for 30 min and then washed in PBS for 1 to 2 min.

Identification of positive cells. A positive result was indicated by the presence of two or more intact cells exhibiting specific fluorescence. A single positive cell required reexamination by a supervisor prior to being reported as positive. A negative result was indicated by the absence of fluorescence in a minimum sampling of 20 ciliated epithelial cells. Samples containing fewer than 20 ciliated epithelial cells were considered inadequate. The staining pattern varied with the infecting virus and the stage of growth. Fluorescent staining was nuclear and/or cytoplasmic and often punctate; it was bright apple green for fluorescein-labeled antibodies and reddish gold for rhodamine-labeled antibodies.

Virus isolation. For viral culture, aliquots of samples were obtained prior to centrifugation to pellet cells or the addition of PBS to remove mucous. A total of 940 samples (141 RS positive and 799 RS negative) were inoculated into rhesus monkey kidney, MRC-5, and A549 cell cultures (Viromed Laboratories, Minneapolis, Minn.; Intracel, Issaquah, Wash.; and/or BioWhittaker, Cockeysville, Md.), incubated at 35°C in a rotating drum for 2 to 3 weeks, and examined for cytopathic effects daily during the first week, and then every other day, and for hemadsorption on days 2, 7, and 14. Isolates were identified by immunofluorescence, using the reagents described above.

Statistical analysis. McNemar's test was used for comparisons of results between test methods.

RESULTS

Comparison of SimulFluor RS reagent with single or dual DFA reagents. After staining, 126 (7.5%) samples were found to have inadequate numbers of ciliated respiratory epithelial cells for performance of DFA. In total, 1,531 samples were evaluated with RS reagent and at least one other DFA reagent, depending on the virus(es) suspected and physician requests (Table 1). Therefore, the number of samples examined for each virus varied.

For RSV, influenza B virus, and adenovirus, the results were identical. For influenza A virus and parainfluenza virus there were 11 discrepancies, but the differences between the reagents were not statistically significant. Overall, there was an agreement of 99.3% between the RS and the single or dual reagents.

Comparison of SimulFluor RS reagent with virus isolation. A total of 940 samples (141 RS positive and 799 RS negative) were tested by RS DFA and culture (Table 2) based on physician requests. Culture was usually requested for specimens from compromised hosts and other seriously ill patients, for lower respiratory tract samples, and for DFA-negative specimens.

RS DFA was significantly more sensitive than culture ($P = 0.001$). The one culture-positive sample missed by RS DFA was obtained from the BAL fluid of a human immunodeficiency virus-infected adult from whom cytomegalovirus (CMV) was also isolated. Only 25 (10.8%) of 232 samples that were influenza A virus positive by RS DFA were cultured during the study period (Table 2). In contrast, 915 samples that were negative for influenza A virus by RS DFA were cultured. RS DFA and culture did not differ in sensitivity for influenza A virus, influenza B virus, or parainfluenza virus ($P = 0.739$, 1.000, and 0.564, respectively). However, culture detected significantly more positive adenovirus samples than did RS DFA ($P = 0.005$).

Additional viruses detected only by culture. Viruses detected only by culture are shown in Table 3. Only 15 (1.9%) of 799 RS DFA-negative samples yielded a respiratory virus included in the antibody pool on culture. For three specimens with dual respiratory virus infection, the second virus was missed by RS DFA and detected only by culture. Thirteen of these samples were collected from adults, and five from children. The RS DFA false-negative specimens included 12 swab samples, 4 aspirates, and 2 BAL fluids. In addition, 66 viruses not included in the RS reagent were detected by culture.

Effect of sample type on DFA results. Of 746 NP aspirates submitted, 5% were inadequate for DFA, and 41% of the remaining samples were positive. Of 787 NP swabs tested, 8% were inadequate, and 32% of the remaining specimens were

TABLE 2. Comparison of SimulFluor RS DFA with culture

Virus	(%) No. of positive samples			*P* value[a]
	Total ($n = 940$)	By RS	By culture	
RSV	77	76 (99)	45 (58.4)	0.001
Influenza A virus	30	25 (83.3)[b]	26 (86.7)	0.739
Influenza B virus	18	15 (83.3)	15 (83.3)	1.000
Parainfluenza virus types 1 to 3	20	19 (95)	18 (90)	0.564
Adenovirus	19	11 (57.9)	19 (100)	0.005
Total	164	146 (89)	123 (75)	0.003

[a] Determined by McNemar's test.
[b] Only 25 of 232 (10.8%) specimens positive for influenza A virus by RS were cultured.

TABLE 3. Viruses detected only by culture

Antibody present in RS reagent	Virus isolated	No. (%) of additional virus isolates from:		Total no. (%) of additional viruses detected (n = 940)
		RS DFA-negative samples (n = 799)	RS DFA-positive samples (n = 141)	
Yes	RSV	1	0	1
	Influenza A virus	5	0	5
	Influenza B virus	2	1[a]	3
	Parainfluenza virus types 1–3	1	0	1
	Adenovirus	6	2[a,b]	8
	Total	15 (1.9%)	3 (2.1%)	18 (1.9%)
No	Rhinovirus	13	0	13
	CMV	22	2[c]	24
	Herpes simplex virus type 1	26	2[a]	28
	Enterovirus 71		1[a]	1
	Total	61 (7.6%)	5 (3.5%)	66 (7%)

[a] RS DFA positive for RSV.
[b] RS DFA positive for parainfluenza virus.
[c] RS DFA positive for influenza A virus.

positive. In contrast, 32% of 44 throat swab samples were inadequate, and only 4 (13%) were positive by DFA.

Forty-six BAL fluid samples were tested by DFA and culture. Of these, one (2%) was inadequate and four (9%) were positive (two for RSV and two for influenza A virus). Two of the DFA-positive samples (one for RSV and one for influenza A virus) were culture negative. All BAL fluid samples were also inoculated into cultures. One RSV isolate and one adenovirus isolate were recovered from 2 of the 41 DFA-negative BAL fluid samples. Other sample types tested, including tracheal aspirates, pleural fluid, and lung biopsy specimens, were negative by both DFA and culture for the seven respiratory viruses detected by the RS reagent.

DISCUSSION

In our study, SimulFluor RS was equivalent in sensitivity to single and dual DFA reagents, allaying concerns that an interference or dilution effect might occur with pooled antibodies or that the shortened incubation period used with the RS would be suboptimal. Furthermore, SimulFluor RS DFA was as sensitive as culture for detection of influenza A and B viruses and parainfluenza virus types 1 to 3. SimulFluor RS DFA was significantly better for detection of RSV than was RhMK, MRC-5, or A549 cell culture. Due to the poor quality of commercially available HEp-2 cells, they are no longer maintained in our laboratory and thus were not inoculated. Only for adenovirus was culture significantly more sensitive than DFA, as previously reported (10). Unfortunately, the other rapid diagnostic test for adenovirus, shell vial centrifugation culture, requires up to 4 to 5 days for optimal sensitivity (8, 11).

The ability to prepare and examine one cell spot and detect RSV, influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus was a great benefit. For all negative specimens and for RSV-positive samples, which stained reddish gold, only a single cell spot was needed. For other DFA-positive samples, the infected cells stained green; then a second slide was stained and read, which took an additional 20 to 35 min. In our study, only 20% of samples (all fluorescein-labeled positives) required more than the initial cell spot for identification. With the use of dual SimulFluor reagents, using just two cell spots, these viruses could be identified as influenza A or B virus, parainfluenza virus, or adenovirus. Oftentimes the staining pattern was a clue, in that influenza virus antigens are expressed in both the nucleus and cytoplasm whereas parainfluenza virus-infected cells show only cytoplasmic staining. Thus, only one additional cell spot was usually stained. In contrast, screening for seven viruses by the use of single or dual DFA reagents would necessitate staining and reading three to seven cell spots for each sample.

Other advantages of DFA are that single or multiple samples can be run, that samples received throughout the day and evening can be tested, and that results can be made available within 1 to 4 h of sample receipt, depending on the clinical need. For all specimens processed during the study period, the average time to reporting of results was 2.25 h. This required a significant reallocation of technical effort but was of tremendous benefit, especially for efficient bed utilization for new admissions from the emergency room and for management of immunocompromised hosts. During the study period, respiratory infections due to RSV, adenovirus, parainfluenza virus, or influenza virus were all diagnosed rapidly in compromised hosts, allowing for prompt institution of therapy when indicated. Previous reports of parainfluenza virus infections in bone marrow transplant recipients have noted a median delay of 9 to 11 days prior to diagnosis and treatment (15). Finally, only with DFA can a sample be examined and assessed as suboptimal and the clinician be notified to recollect the specimen.

The limitations of DFA include the possibility of inadequate numbers of respiratory epithelial cells for examination and the need for experience in the preparation and reading of cell smears. The use of a cytocentrifuge to prepare slides is beneficial in both areas. We and others have found that cytospin preparation reduces the number of inadequate slides and improves the morphology and readability of the stained preparations (1, 6). The additional cost is approximately $1 per slide for the cytofunnel.

It should be noted that the SimulFluor reagents used did not differentiate among parainfluenza virus types 1, 2, and 3, and no requests for typing were received from clinicians. However, all lower respiratory tract specimens were cultured, and parainfluenza virus isolates were routinely typed. Of note, parain-

fluenza virus type 4, which can cause disease, especially in compromised hosts (12), is not included in the SimulFluor pools but can be recovered by culture.

DFA testing also necessitates that the clinical laboratory develop its own training program since the companies selling the antibodies do not provide color photographs of clinical samples, showing both specific and nonspecific staining patterns, to help train users. The staff must develop expertise in identifying the morphology of ciliated respiratory epithelial cells, in assessing the inadequacy of cell smears, and in distinguishing specific from nonspecific results for all the viruses tested. This requires a substantial commitment for training and continuing quality control. Retaining stained slides at 4°C for reexamination after culture results become available is very helpful for this purpose.

In this report, only 18 additional respiratory viruses were recovered from 940 cultured samples (1.9%). In many of these specimens, the number of ciliated respiratory epithelial cells was deemed inadequate on reexamination. As expected, adenovirus was disproportionately represented in the DFA-negative, culture-positive samples (8 of the 18). Of the 66 other viruses recovered, predominantly herpes simplex virus type 1, CMV, and rhinovirus, culture may have provided useful information for patient management. Thus, in our laboratory, culture is always performed in addition to DFA for specimens from immunocompromised hosts and patients with lower respiratory tract disease and whenever adenovirus is suspected.

In the future, we hope to further improve DFA results by devoting more effort to improving collection of samples, especially from adults, who shed lower titers of virus (7). NP aspirates, collected predominantly from infants and young children, yielded more positives and fewer inadequate samples than the other specimen types. The majority of screen-negative, culture-positive samples in this study were swab specimens collected from adults. Throat swabs in particular tended to provide an inadequate number of respiratory epithelial cells, and their use for DFA should be discouraged.

In conclusion, SimulFluor RS provides an efficient, economical, and sensitive means of rapid diagnosis of multiple respiratory viruses and can be readily incorporated into a routine clinical laboratory. Samples can be tested singly or in batches as needed. The availability of a rapid DFA screening reagent for detection of multiple common respiratory viruses within 1 to 2 h of sample collection should be of great benefit in terms of patient management and infection control.

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REFERENCES

1. **Doing, K. M., M. A. Jerkofsky, E. G. Dow, and J. A. Jellison.** 1998. Use of fluorescent-antibody staining of cytocentrifuge-prepared smears in combination with cell culture for direct detection of respiratory viruses. J. Clin. Microbiol. **36:**2112–2114.
2. **Engler, H. D., and J. Preuss.** 1997. Laboratory diagnosis of respiratory virus infections in 24 hours by utilizing shell vial cultures. J. Clin. Microbiol. **35:**2165–2167.
3. **Hayden, F. G., A. D. Osterhaus, J. J. Treanor, D. M. Fleming, F. Y. Aoki, K. G. Nicholson, A. M. Bohnen, H. M. Hirst, O. Keene, and K. Wightman.** 1997. Efficacy and safety of the neuraminidase inhibitor zanamivir in the treatment of influenza virus infections. GG167 Influenza Study Group. N. Engl. J. Med. **337:**874–880.
4. **Jiang, F., K. J. Henrickson, and L. L. Savatski.** 1998. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B and human parainfluenza virus types 1, 2 and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). Clin. Infect. Dis. **26:**1397–1402.
5. **Landry, M. L.** 1997. Rapid viral diagnosis, p. 608–617. *In* N. R. Rose, E. Conway de Macario, J. D. Folds, H. C. Lane, and R. M. Nakamura (ed.), Manual of clinical laboratory immunology, 5th ed. ASM Press, Washington, D.C.
6. **Landry, M. L., D. Ferguson, and J. Wlochowski.** 1997. Detection of herpes simplex virus in clinical specimens by cytospin-enhanced direct immunofluorescence. J. Clin. Microbiol. **35:**302–304.
7. **Landry, M. L., S. Cohen, and D. Ferguson.** 2000. Impact of sample type on rapid detection of influenza virus by cytospin-enhanced immunofluorescence and membrane enzyme-linked immunosorbent assay. J. Clin. Microbiol. **38:** 433–434.
8. **Mahafzah, A. M., and M. L. Landry.** 1989. Evaluation of conventional culture, centrifugation culture and immunofluorescent reagents for the rapid diagnosis of adenovirus infections. Diagn. Microbiol. Infect. Dis. **12:**407–411.
9. **Matthey, S., D. Nicholson, S. Ruhs, B. Alden, M. Knock, K. Schultz, and A. Schmuecker.** 1992. Rapid detection of respiratory viruses by shell vial culture and direct staining by using pooled and individual monoclonal antibodies. J. Clin. Microbiol. **30:**540–544.
10. **Minnich, L., and C. G. Ray.** 1980. Comparison of direct immunofluorescent staining of clinical specimens for respiratory virus antigens with conventional isolation techniques. J. Clin. Microbiol. **12:**391–394.
11. **Olsen, M. A., K. M. Shuck, A. R. Sambol, S. M. Flor, J. O'Brien, and B. J. Cabrera.** 1993. Isolation of seven respiratory viruses in shell vials: a practical and highly sensitive method. J. Clin. Microbiol. **31:**422–425.
12. **Rubin, E. E., P. Quennec, and J. C. McDonald.** 1993. Infections due to parainfluenza virus type 4 in children. Clin. Infect. Dis. **17:**998–1002.
13. **Stout, C., M. D. Murphy, S. Lawrence, and S. Julian.** 1989. Evaluation of a monoclonal antibody pool for rapid diagnosis of respiratory viral infections. J. Clin. Microbiol. **27:**448–452.
14. **Wendt, C. H., and M. I. Hertz.** 1995. Respiratory syncytial virus and parainfluenza virus infections in the immunocompromised host. Semin. Respir. Infect. **10:**224–231.
15. **Wendt, C. H., D. J. Weisdorf, M. C. Jordan, H. H. Balfour, Jr., and M. I. Hertz.** 1992. Parainfluenza virus respiratory infection after bone marrow transplantation. N. Engl. J. Med. **326:**921–926.
16. **Whimbey, E., L. S. Elting, R. B. Couch, J. Englund, K. Rolston, I. Raad, Q. Nguyen, C. Ippoliti, and M. Luna.** 1994. Influenza A virus infection among hospitalized adult bone marrow transplant recipients. Bone Marrow Transplant. **13:**437–440.