Evaluation of Direct Immunofluorescence, Enzyme Immunoassay, Centrifugation Culture, and Conventional Culture for the Detection of Respiratory Syncytial Virus

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Four methods of detecting respiratory syncytial virus (RSV) from clinical specimens were evaluated. A total of 410 specimens consisting of nasopharyngeal washes, aspirates, and swabs were simultaneously tested for the presence of RSV by direct immunofluorescence assay (DFA), enzyme immunoassay (EIA) (Kallestad Pathfinder), shell vial centrifugation culture (SVC), and conventional culture. DFA identified 146 (83%) of the 175 positive cases, EIA detected 153 (87%), SVC detected 127 (73%), and conventional culture detected 70 (40%). Conventional culture isolated an additional 19 respiratory viruses other than RSV. DFA and EIA were able to detect nonviable virus not isolated by a culture method, and SVC isolated low-titer virus not detected by conventional culture. DFA and EIA gave similar results; however, the EIA system was less dependent on technical expertise. The use of SVC enhanced the conventional culture system with 63 RSV isolates not recovered from the tube culture. We recommend complementary use of both culture and nonculture methods in the detection of RSV.

Seasonal rises in respiratory syncytial virus (RSV) cause outbreaks of acute lower respiratory tract infections that can be severe in the young and immunocompromised patient (2, 5). The increased use of ribavirin for the treatment of RSV has mandated the use of accurate, rapid laboratory procedures (3, 5, 11, 13, 15). The success of these procedures, however, depends on many variables. The accuracy of results is dependent upon the collection of adequate and appropriate specimens (1, 2, 4, 5, 7, 8, 17, 19), as well as diligent specimen handling and transportation to the laboratory. Furthermore, tests subject to interpretation require technical expertise (4, 5, 20).

It has been demonstrated that RSV is a very labile virus and that it loses its viability in transit (5, 12). To overcome this limitation, immunofluorescent staining of direct smears (DFA) has become a standard means of RSV detection (13, 19, 20). This test is limited, however, by the subjective nature of reading the stained smear and the possibility of false-positive results occurring in the late stage of the disease (1, 2, 4, 5, 20).

The use of enzyme immunoassay (EIA) for the direct detection of RSV antigen is becoming readily available for the hospital laboratory as well as the small clinic laboratory. EIAs require laboratory support but little technical knowledge to interpret, and, like DFA, they can detect both viable and nonviable antigen (1, 2, 4, 13, 17, 20).

Shell vial centrifugation cultures (SVC) have been used extensively in the detection of a variety of viruses, most notably cytomegalovirus, herpes simplex virus, and influenza virus (6, 9, 10, 14, 16). SVC to detect RSV is not as rapid as DFA or EIA, but it will detect low-titer virus, which can be missed by the other methods.

In this study, four methods of detection were used to maximize the diagnosis of (RSV) from clinical specimens. We chose to use both culture and nonculture methods of detection because of the labile nature of the virus and the distance some specimens had to travel before they arrived at the laboratory. Viral isolation in conventional tissue culture tubes and in centrifugation cultures was complemented with the use of DFA and an EIA system (Kallestad Pathfinder; Kallestad Diagnostics, Chaska, Minn.).

MATERIALS AND METHODS

Specimens. Specimens were collected from hospitalized and nonhospitalized pediatric patients in northeastern Wisconsin. There were 340 (83%) nasopharyngeal washings, 29 (7%) nasopharyngeal aspirates, and 41 (10%) nasopharyngeal swabs submitted to the laboratory for RSV detection. Nasopharyngeal swabs were taken by inserting a dry calcium alginate, aluminum-shafted swab into the nasopharyngeal area. The swab was allowed to remain in the area for 10 to 30 s and then rotated and withdrawn. This was done twice. One swab was used to prepare the direct smear at the bedside, and then both swabs were placed in viral transport medium. The viral transport medium was prepared in house and contained Eagle minimum essential medium, 10% gelatin, and antibiotics.

Nasopharyngeal washes were collected by instilling 2 to 5 ml of sterile saline into the nostril and aspirating it into a tube of viral transport medium. Nasopharyngeal aspirates were taken by using a French soft plastic feeding tube to suction secretions from the nasopharyngeal area. The secretions were then combined with viral transport medium. Specimens from outside the hospital were held at 4°C until transported to the laboratory. The time from specimen collection to laboratory inoculation ranged from 1 to 8 h depending on the location of the outlying clinic. In-house specimens were transported to the laboratory immediately upon collection (time to arrival at laboratory, less than 6 min).

Direct smears. Direct smears were prepared in the laboratory from nasopharyngeal washes and aspirates by centrifuging the specimen at 700 × g for 10 to 15 min and applying part of the resulting pellet onto a two-ringed slide. The remaining portion of the pellet was resuspended in viral transport medium for inoculation for EIA, SVC, and con-

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ventional culture. Direct smears from nasopharyngeal swabs were prepared at the bedside. Once air dried, the slides were fixed in acetone for 10 min and air dried before staining. A direct RSV fluorescent antibody stain was used (Difco Laboratories, Detroit, Mich., and Baxter Healthcare Corp./Bartels Diagnostic Div., Issaquah, Wash.) on the smears. Enough stain (approximately 25 to 50 µl) was added to cover the ringed area of the slide. The slide was then incubated at 35°C in a humidity chamber for 30 min after which it was washed twice in phosphate-buffered saline (PBS) for 5 min. Excess moisture was blotted from the slide, and a buffered glycerol mounting fluid was applied for placement of the coverslip. The slides were viewed at 400 x on a fluorescence microscope.

EIA. The Kallestad Pathfinder direct antigen detection system (EIA) for RSV (Kallestad Diagnostics, Chaska, Minn.) was processed in parallel with the direct smear. The system used polystyrene reaction tubes precoated with rabbit anti-RSV immunoglobulin G. In accordance with the manufacturer's instructions, 100 µl of horseradish peroxidase-conjugated murine monoclonal antibody and 1 drop of assay treatment buffer were added to the reaction tubes. The specimen was vortexed, and 300 µl of the specimen was added to the reaction tube. Controls were inoculated in the same manner in accordance with the instructions. The tubes were gently mixed and incubated for 60 min at room temperature. After this time, the specimen was aspirated from the tube and the tube was washed six times with approximately 4 ml of distilled water. Excess moisture was removed by inverting the tube, shaking it, and blotting the rim. A working color reagent (tetramethylbenzidine) was prepared, and 50 µl was added to each tube. The tubes were incubated in the dark at room temperature for 15 min. The tubes were then read visually against a white background for a blue color indicating positive detection of RSV antigen.

SVC. After the medium was aspirated, samples were vortexed and inoculated (0.25 ml) into two HEP-2 shell vials. These vials were prepared in house twice weekly and were typically less than 4 days old and 80% confluent when inoculated. The vials were then centrifuged at 700 x g for 60 min. The vials were then supplemented with 1.0 ml of medium containing Eagle minimum essential medium, 5% fetal bovine serum, and antibiotics, incubated at 35°C for 24 or 48 h, and stained with the same monoclonal antibody used in evaluating the direct smear.

Staining of the coverslip was completed by first aspirating the medium from the vial and adding 1 ml of acetone for 10 min. The acetone was then aspirated, and the vial was rinsed with 2 ml of PBS. The PBS was then decanted, and enough stain was added to cover the coverslip in the shell vial. The vial was covered and incubated at 35°C for 30 min and then rinsed twice for 5 min with PBS. The coverslips were removed, mounted on a slide with buffered glycerol mounting fluid, and read at 400 x on a fluorescence microscope. The second shell vial was stained and read after 2 days of incubation if the first shell vial was negative after staining at 24 h.

Conventional culture. The final portion of the resuspended specimen was divided into aliquots and inoculated into two HEP-2 culture tubes and one primary rhesus monkey kidney tube (Viromed Laboratories, Minneapolis, Minn.). The HEP-2 tubes were grown in house twice weekly and were generally no older than 4 days postseeding and 80% confluent. The HEP-2 tubes contained approximately 2 ml of medium containing Eagle minimum essential medium, 5% fetal bovine serum, and antibiotics, and rhesus monkey kidney tubes contained approximately 2 ml of the same medium without the fetal bovine serum. The tubes were incubated on roller drums in a CO2 incubator at 35°C and observed every other day for 14 days or until the samples became positive. Hemagglutination tests were performed on the rhesus monkey kidney cells twice in the course of their evaluation or in the presence of cytopathic effect suggesting the presence of influenza or parainfluenza. Positive isolates were confirmed by direct or indirect immunofluorescence by using monoclonal antibodies specific for the isolate.

RESULTS

Overall, 410 clinical specimens were subjected to RSV antigen detection by DFA, EIA, SVC, and conventional culture. The total number of specimens negative for RSV by all four methods was 235. The other 175 specimens were positive for RSV by one or more of the tests. There were a total of 133 culture- or SVC-positive specimens and an additional 42 that were positive only by a nonculture method.

Only 21 of the 175 RSV-positive specimens were detected by only one method. The majority of positive specimens were detected by three or four tests (Table 1). One-quarter (43) of the positive RSV results were acquired from a combination of only two methods.

Table 2 shows the number of positive results for each method, expressed as percentages of the total number of specimens tested and of the total number of positive results.

SVC isolated 57 cases of RSV that were not isolated by conventional culture. There were 48 cases of RSV that were not isolated by SVC. Forty-two of these were not detected in conventional culture either.

There were 146 RSV cases detected by DFA. This gave DFA a sensitivity of 84% (112 of 133) and a specificity of 88% (243 of 277) when compared to a combination of the culture-

<table>
<thead>
<tr>
<th>TABLE 1. Concordant and discordant positive RSV test results*</th>
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<tr>
<td>No. of tests</td>
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<tr>
<td>--------------</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
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<td>EIA</td>
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<td>DFA</td>
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* Total number of specimens = 410; total number of positive specimens = 175.

TABLE 2. Detection of RSV by various methods

<table>
<thead>
<tr>
<th>Method*</th>
<th>No. of positive specimens</th>
<th>410 specimens (total)</th>
<th>175 specimens (positive by any test)</th>
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</thead>
<tbody>
<tr>
<td>DFA</td>
<td>146</td>
<td>36</td>
<td>83</td>
</tr>
<tr>
<td>EIA</td>
<td>153</td>
<td>37</td>
<td>87</td>
</tr>
<tr>
<td>SVC</td>
<td>127</td>
<td>31</td>
<td>73</td>
</tr>
<tr>
<td>CC</td>
<td>70</td>
<td>17</td>
<td>40</td>
</tr>
<tr>
<td>SVC plus CC</td>
<td>133</td>
<td>32</td>
<td>76</td>
</tr>
</tbody>
</table>

* DFA, Direct smear; EIA, Kallestad RSV direct antigen detection system; SVC, shell vial centrifugation culture; CC, conventional tube culture.
positive samples. DFA was positive in five cases in which none of the other three tests was positive. The presence of one or more cells displaying characteristic cytoplasmic fluorescence was considered a DFA-positive result. Three DFA specimens that had fewer than 10 columnar epithelial cells were removed from the study. In these three cases, the corresponding EIA, SVC, and conventional cultures were all negative.

A total of 153 EIAs were positive. Eight of these results could not be confirmed by one of the other three methods. A blocking antibody was not used on these specimens. There were 138 EIA-positive results that were confirmed by DFA and an additional 7 that were confirmed by a culture method. In comparison to the culture methods, EIA had a sensitivity of 87% (116 of 133) and a specificity of 88% (243 of 277). EIA also detected 37 cases that were not confirmed by a culture method. When compared with the DFA-positive results, EIA had a sensitivity of 94% (138 of 146) and a specificity of 94% (249 of 264). However, 15 EIA-positive cases were not positive by DFA.

Conventional culture detected an additional 19 specimens positive for viruses other than RSV. There were six influenza A, nine adenovirus, and four paramyxovirus isolates. The conventional culture did not detect any RSV isolates that had not been previously detected by one of the other three methods.

**DISCUSSION**

The need for complementary use of culture and nonculture methods of RSV detection has been well documented (1, 4, 5, 13, 19). Not only can nonculture methods detect virus which has lost viability in transit (2), but the results obtained from these tests are typically more rapid than those provided by a culture method. Culture methods, on the other hand, are more favorable for detecting low titers of viable virus as well as for detecting viruses other than RSV (1, 4, 5, 13, 19).

This study investigated the use of two nonculture and two culture methods of RSV isolation. Because of the critical nature of an appropriate specimen, physicians were encouraged to submit nasopharyngeal washes and aspirates. In some cases, nasopharyngeal swabs were submitted because of the lack of appropriate collection equipment or because of the reduced trauma to the patient. In these cases, two swabs—one for DFA and a combination of the two collected swabs for EIA, SVC, and conventional culture—were required to negate the loss of viral particles during slide preparation.

To increase the sensitivity of the culture methods, HEp-2 cells were prepared in house twice weekly. The cells were used before they reached a complete monolayer, thus increasing the chances of observing the cytopathic effect of the RSV syncitia (18). HEp-2 cells have been demonstrated to be the most sensitive cell line for isolating RSV, but they tend to quickly overgrow, thus reducing the sensitivity to RSV (18).

To complement the isolation of RSV in conventional tubes, HEp-2 cells were used in SVCs. This method was superior to the conventional culture for detecting viable RSV. There were 63 RSV isolates from SVC that were not detected in conventional tube culture. Only six cases had a positive conventional culture result and a negative SVC result. All six of these cases were also positive by DFA and EIA. These discrepancies could be accounted for on the basis of unequal distribution of the original inoculum.

In further support of the use of SVC in isolating RSV, there were eight SVC isolates that were not detected by conventional culture, DFA, or EIA. These were presumably low-titer specimens that required the enhancement of centrifugation for isolation.

The results of the DFA data are twofold. There were 112 RSV-positive smears that were confirmed by isolation in a culture system. An additional 29 were culture negative but confirmed with positive EIA results. There were also five cases in which DFA was the only positive test of the four tests performed. It has been reported (1) that false-positive results may occur in DFA when the specimen is collected at a late stage of the disease. False-positive results can also be reported when nonspecific fluorescence caused by excess mucus or fluorescing neutrophils are read as positive by an inexperienced technologist (5).

The use of DFA requires technical expertise and a smear containing columnar epithelial cells. The advantage of the test is that the results are rapid and can provide valuable, timely information to the physician.

The EIA system is also rapid and does not require technical expertise to run. The Kallestad EIA system proved to be not only sensitive but easy to perform and interpret. This system used coated reaction tubes, which were easy to handle and store. There was no additional large equipment such as a bulky washer or reader needed for the test. The blue color of the positive reaction was quite easy to visualize, and the lack of a filtration device remedied problems experienced in the use of other EIA kits (20).

The EIA RSV-positive results were confirmed by DFA or by a culture method in all but eight cases. Unfortunately, a blocking antibody was not used to confirm these results and would have been helpful in interpreting the specificity of the test. It has been reported that EIA is more sensitive than culture (17). We found that EIA was able to detect both culture-confirmed virus and virus we considered nonviable on the basis of positive-DFA/negative-culture results. EIA and DFA were run on an as-needed basis, so batching of the testing was prohibited. Batching would make this method more cost effective to use.

The conventional culture method did not produce as many positive isolates as any of the other methods. This seems to indicate that it should not be used alone for the detection of RSV, especially if there is a large transit time between specimen collection and receipt. There were no conventional culture isolates that were not detected by one or more of the other methods. This method did, however, isolate other respiratory viruses which can cause clinical symptoms similar to those caused by RSV. Because SVC, EIA, and DFA are specific to a single virus being investigated, combining conventional culture (with more than one cell line) with the rapid tests is imperative.

In conclusion, these data suggest that the use of a single test for RSV is inappropriate. There exists a need for rapid, sensitive testing of both viable and nonviable virus. The Kallestad EIA system provided results comparable to those of DFA while requiring a similar time and less technical expertise. SVC proved superior to conventional culture in isolating RSV but was limited to identifying virus specific to the stain being used and not other respiratory viruses. The limitations of cell culture do not outweigh the need to support rapid techniques with the ability to make differential as well as low-virus-titer detections.

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LITERATURE CITED


