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Detection of Respiratory Syncytial Virus in Nasopharyngeal Secretions by Shell Vial Technique

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A shell vial technique was used to recover respiratory syncytial virus (RSV) from frozen nasopharyngeal specimens previously tested by rapid diagnostic methods. With specimens determined to be positive by direct fluorescence assay (DFA), the shell vial technique was at least as sensitive as conventional tissue culture (92 versus 90%). The majority of RSV isolates were detected within 16 h postinoculation, versus an average of 4.5 days by conventional techniques. Also, the shell vial method recovered RSV from 16 of 17 specimens (94%) which had previously tested positive by enzyme immunoassay (EIA). In addition, the shell vial method detected RSV in 4 and 11% of specimens previously determined to be negative by DFA and EIA, respectively. Therefore, we recommend the use of the shell vial technique for specimens testing negative by the rapid methods of DFA or EIA.

Respiratory syncytial virus (RSV) is a major cause of acute respiratory illness in infants and young children, being responsible for 50% of all bronchiolitis cases and 25% of all pneumonia cases during the first months of life (12). With the availability of effective therapy for RSV infections and for the prevention of nosocomial infection, rapid, sensitive, and specific laboratory tests for RSV are desirable. Current methods for the detection of RSV include viral isolation in tissue culture, immunofluorescence assay (IFA), and enzyme immunoassay (EIA). Tissue culture is still considered to be the standard against which all other methods are compared (16). Due to slow detection, the tissue culture system has been largely replaced by rapid techniques such as IFA and EIA. When compared to tissue culture, however, IFA and EIA had sensitivities and specificities ranging from 41 to 97% in a number of studies (5, 11, 16).

A modification of the conventional tissue culture, using low-speed centrifugation in shell vial culture tubes, was first described by Gleaves and colleagues for the rapid detection of cytomegalovirus in urine specimens (6). Subsequently, this technique has become an important adjunct to conventional tube culture methods by improving the rate of detection of and overall sensitivity with several viruses (7–9). In this study, we first compared the shell vial technique to conventional tissue culture by using HEp-2 cells for the detection of RSV from specimens that had previously tested positive by direct fluorescence assay (DFA). We further tested for the presence of RSV by the shell vial technique using specimens that had previously tested negative by DFA and both positive and negative by EIA.

MATERIALS AND METHODS

Cell cultures. HEp-2 cells (CCL 23) obtained from the American Type Culture Collection, Rockville, Md., were maintained in minimum essential medium with Earle balanced salt solution (M. A. Whittaker Bioproducts, Walkersville, Md.) supplemented with 10% fetal bovine serum (FBS)

(GIBCO, Gaithersburg, Md.), 2 mM glutamine, 2 μg of amphotericin B per ml, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml (MEM–10% FBS). Tissue culture tubes and shell vials were seeded with 1.5 and 1 ml, respectively, of HEp-2 cells at a concentration of 10^5/ml and incubated for 2 days at 35°C prior to specimen inoculation.

Specimens. Clinical specimens were collected from Rainbow Babies and Children's Hospital, Cleveland, Ohio, from January through April 1989. Nasopharyngeal aspirates were collected by inserting a 5-French nasogastric feeding tube into the nasopharynx and aspirating with a syringe. The aspirates were expelled into viral transport medium and delivered to the laboratory. Each specimen was vigorously vortexed for 10 s, followed by centrifugation at 1,400 × g for 5 min to pellet the epithelial cells. The cell pellet was washed twice with phosphate-buffered saline (PBS) and tested by DFA. The supernatants from some of the specimens were tested for RSV by EIA. The remainder of the supernatants were stored frozen at −70°C until further use in this study.

DFA. Fluorescein isothiocyanate-conjugated RSV monoclonal antibody was purchased from Bartels Immunodiagnostic Supplies, Inc., Bellvue, Wash. The DFA procedure followed the recommendation of the supplier. Briefly, the patient specimen was centrifuged and washed twice in PBS. One drop of the resulting cellular pellet was spread on a 10-mm circle of a microscope slide. The slide was air dried and fixed in cold acetone for 10 min. The cell smear was overlaid with 25 μl of Bartels RSV DFA stain and incubated in a humid chamber for 30 min at 35°C. The slide was then washed with PBS, air dried, and examined microscopically. A specimen negative for RSV was one in which at least 10 epithelial cells with no fluorescence were seen. Specimens with fewer than 10 cells, with no fluorescence, were rejected for DFA analysis. Specimens showing one or more cells with characteristic cytoplasmic fluorescence were considered to be positive for RSV.

EIA. The EIA kit used was an RSV TestPack purchased from Abbott Laboratories, North Chicago, Ill., and used according to the supplier's recommendations. Briefly, 0.75 ml of a patient sample collected in viral transport medium

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was treated with sample treatment buffer and then filtered to clarify the specimen. Microparticles coated with bovine RSV antibody were added to the specimen simultaneously with biotin-labeled bovine RSV antibody. After 10 min of incubation at room temperature, the solution was transferred to a reaction disk with alkaline phosphatase-labeled antibiotin antibody and washed, and substrate was added. Specimens which produced the image of a plus sign were considered to be positive for RSV antigen, while specimens which produced the image of a minus sign were considered to be negative.

Inoculation of specimens. (i) Conventional cell culture tubes. Prior to specimen inoculation, the medium in the culture tubes was discarded and replaced with 1.5 ml of fresh MEM–2% FBS. A 0.2-ml sample of each specimen was then inoculated into each tube, incubated at 35°C with 4% CO_2 in a stationary rack, and observed daily up to 14 days for cytopathic effect (CPE). In cultures negative for CPE at day 7, the medium was exchanged with fresh MEM–2% FBS. Infected cells were scraped from the tube, washed twice with PBS, centrifuged, applied to a microscope slide, allowed to air dry, fixed in cold acetone for 10 min, and stained as described above.

(ii) Shell vials. A modification of the procedure used by Gleaves et al. was followed for the shell vial technique (8). Briefly, the medium from the shell vials was aspirated and 0.2 ml of specimen was added directly to each of three shell vials. The vials were recapped and centrifuged at 700 × g for 60 min at 32°C. After centrifugation, 1.0 ml of MEM–2% FBS was added to each vial, the caps were replaced, and the vials were incubated at 35°C. The vials were examined daily for typical CPE before being stained for RSV at 16, 40, and 64 h. Prior to being stained, the coverslips were washed twice with PBS and fixed in cold acetone for 10 min.

RESULTS

To compare the sensitivities of the shell vial and conventional cell culture assays for detection of RSV, specimens that had originally tested positive by DFA were used. By selecting these specimens, we expected a high RSV recovery rate for comparing the sensitivities of the two culture techniques. Each test specimen was inoculated into three shell vial cultures and one tube culture. One shell vial was incubated for 16 h, one was incubated for 40 h, and one was incubated for 64 h. The shell vials were checked for CPE before being stained in the DFA. The tube culture was incubated for up to 14 days and examined daily for CPE. Tube cultures showing CPE were confirmed for RSV by DFA.

From 50 clinical specimens that had originally tested positive for RSV by DFA, RSV was recovered from 46 shell vial cultures compared with 45 tube cultures. Microscopic examinations of the shell vials were negative for CPE over the 3-day incubation period. RSV was detected in 43 of the 50 specimens at 16 h, 3 more at 40 h, and no additional vials at 64 h. A total of 92% of the original DFA-positive specimens were detected by the shell vial technique using HEp-2 cells.

In the conventional tube cultures, CPE was detected in 45 of the 50 specimens, for a 90% recovery. These cultures were confirmed for RSV by DFA. The CPE development took from 2 to 8 days, with an average of 4.5 days. In a direct comparison between the shell vial and the conventional tissue culture, RSV was detected in 45 of 50 specimens by

TABLE 1. HEp-2 shell vial cultures inoculated with clinical specimens previously tested by rapid methods

Test result	No. (%) of results of shell vial detection of RSV	
	Positive	Negative
IFA[a] positive	46 (92)	4 (8)
IFA negative	6 (4)	141 (96)
EIA positive	16 (94)	1 (6)
EIA negative	3 (11)	25 (89)

[a] Shell vial cultures were stained in the IFA for RSV at 16, 40, and 64 h.

both culture techniques. One additional specimen was detected by shell vial but missed by conventional culture.

The sensitivity of the shell vial method was tested further with 147 clinical specimens that had originally tested negative for RSV by DFA. As shown in Table 1, RSV was detected in six of these specimens after 16 h of incubation. Further incubation produced no additional positive results.

Since specimens analyzed by DFA consist of a cellular pellet and the cultures in this study were performed by using a cell-free supernatant, as was done with EIA, a comparison of the shell vial technique with EIA was performed. The results are shown in Table 1. Again, incubation beyond 16 h detected no additional positive specimens.

DISCUSSION

We have demonstrated that the shell vial technique is superior to conventional cell culture for the detection of RSV by virtue of its rapidity without loss of sensitivity. In fact, the shell vial technique was able to detect one specimen missed by the conventional culture. The majority of RSV isolates (all but three) were detected in shell vials within 16 h, with 100% detected by 40 h.

The average time for detection by conventional culture was 4.5 days, dependent on the observation of CPE. In diagnostic laboratories, conventional cultures are not usually detected by IFA staining only, as in the shell vial. However, in this study, the rapid detection by shell vial may be attributed to the pre-CPE staining, which was not performed with conventional cultures. To test this possibility, we further tested 10 frozen specimens which were previously determined to be positive by DFA. The results showed that in the shell vial, all the specimens tested were positive by 40 h postinoculation, compared with only 80% in conventional culture (data not shown). In addition, the number of infected cells was much higher in shell vials than in conventional cultures; in 2 of the 10 conventional cultures, only 1 infected cell was observed, compared with 20 to 30 infected cells in the shell vial. These results support previous studies by Gleaves et al. (7, 8), in which the shell vial assay was found to be more sensitive than conventional culture for cytomegalovirus and herpes simplex virus, whether both cultures or only the shell vial was stained in the IFA.

Although tissue culture has been used as a standard to which other diagnostic tests have been compared (16), studies comparing the sensitivities of tissue culture and IFA for RSV have yielded conflicting data (3–5, 13). In this study, we show that DFA, the shell vial, and conventional culture are all sensitive for RSV detection, with the shell vial being the most sensitive (Table 1). However, the specimens used for both methods of culture were frozen and thawed, and RSV has been recognized as being extremely labile to

freezing (10). Therefore, the fact that only 94% of the previously tested positive clinical specimens were detected by the shell vial may be partially explained by this potential loss of virus viability.

The Abbott EIA TestPack RSV has been reported to be sensitive, rapid, and easy to perform (13). Although RSV was detected by shell vial culture in 94% of specimens positive by EIA, 11% of the specimens negative by EIA also tested positive. Therefore, we recommend that EIA-negative specimens be cultured by using the shell vial system with results available overnight.

In theory, the sensitivity of each of these detection methods should vary with regard to viral titer. For tissue culture, only one infectious viral particle is necessary to cause infection. Generally for IFA, only one infected epithelial cell is required for a positive determination. Abbott's EIA has been reported to require up to 10 infected epithelial cells from nasal secretions before a positive specimen can be detected (13). Van Dyke and Murphy-Corb have reported the average RSV titer in the nasal fluid of acutely infected infants to be 2×10^4 PFU/ml on the first day of hospitalization (15). Although the sensitivity of each of the three test methods is variable, titers of RSV in clinical specimens are generally high enough to be detected by any of these three methods. With low-viral-titer specimens, though, culture amplification may be required for detection.

In tissue culture systems, the sensitivity of the cell line is extremely important. Prior to this study, we tested several cell lines at 2 days postseeding and 80% confluency for their abilities to recover various titrations of a laboratory stock of RSV (data not shown). The use of HEp-2 cells resulted in the largest number of infected cells, followed by use of CV-1 and A-549 cells. Therefore, for the purpose of comparing the two culture systems in this study, only HEp-2 cells were selected. Arens et al. reported that the use of HEp-2 cells only for RSV detects only about 75% of positive specimens, compared with 100% using a combination of other cell types (1). The lower rate of RSV recovery in the study of Arens et al. may have been due to the age and/or passage of the cells. Treuhaft et al. suggested the use of HEp-2 cells less than 4 days old for RSV (14). In this study, subconfluent cells were used 2 days postseeding, which may have contributed to the high recovery of RSV.

In summary, we have described the successful application of the shell vial technique for the detection of RSV in clinical specimens. We have shown that a number of specimens reported to be negative by IFA and EIA were positive for RSV when tested by tissue culture. Previous studies have reported that up to 20% of RSV-negative specimens processed by rapid screening methods may contain RSV and/or other viruses when tested by tissue culture (2, 13). In view of these findings, we recommend the use of tissue culture which includes the shell vial with HEp-2 cells as an adjunct to rapid screening methods for specimens requested for RSV detection.

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