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Isolation of Seven Respiratory Viruses in Shell Vials: a Practical and Highly Sensitive Method

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The isolation of respiratory viruses in shell vials was compared with isolation in tube cultures in order to determine the sensitivity of the former, rapid method. Twenty of 21 influenza virus and 15 of 15 parainfluenza virus isolates were recovered in shell vials. One hundred twenty-seven of 138 respiratory syncytial virus isolates were detected in shell vials, but only 10 of 21 adenovirus isolates were positive by the rapid method. Shell vials are very effective for the diagnosis of respiratory viral infections, except for those caused by adenovirus.

With the advent of new antiviral agents, it is becoming increasingly important to develop methods for the rapid isolation and identification of respiratory viruses. Numerous rapid antigen detection systems are available for the diagnosis of infections by respiratory syncytial virus (RSV) which are very sensitive and specific (1, 5–7, 14). However, few commercially available kits for the rapid detection of other respiratory viral antigens are on the market (13, 15).

Traditionally, the isolation of the respiratory viruses has been performed with a variety of different cell types by using tube cultures. This method is considered the "gold standard" for the diagnosis of viral respiratory tract infections. Typically, tube cultures are held for up to 14 days and observed for the appearance of cytopathic effect (CPE). In addition, cultures can be screened by hemadsorption for influenza and parainfluenza viruses or by indirect immunofluorescence to detect all of the common respiratory viruses. The disadvantages of tube cultures include the long period before results are available, the many labor-intensive steps during the time that the cultures are held, and the need for highly trained technologists to observe the cultures for CPE.

Isolation in shell vials has been adapted for the detection of respiratory viruses, including influenza A and B viruses, adenovirus, and RSV (3, 4, 7, 9–11). Here, we report the use of this method for the rapid identification of seven viruses from routine respiratory specimens.

Specimens. Nasal or nasopharyngeal wash specimens were collected by physicians primarily from infants and children, whereas throat swabs and/or washes were collected primarily from adults with suspected viral respiratory tract infections. For each subject, the mucosal surface of the nares was first swabbed with a Dacron swab, which was placed in a tube of virus transport medium (2-sucrose phosphate plus gentamicin and amphotericin B [Fungizone]). One to 2 ml of sterile saline was then inserted into the nasal passage with a syringe and tubing, and the saline was withdrawn into the syringe. The washings were expelled into the tube of virus transport medium containing the swab and transported to the laboratory as soon as possible on wet ice. Throat washes were collected by having subjects gargle with small amounts of sterile saline. A tube of virus transport medium was added to the throat gargle, and the specimen was transported to the laboratory as described above. Specimens were held at 4°C when they could not be inoculated into cell culture immediately.

Before the prospective evaluation was initiated, a small study was performed with frozen specimens known to be positive. These positive specimens known to contain influenza A virus, parainfluenza 1, 2, or 3, and adenovirus stored frozen at −70°C were thawed and inoculated into shell vials by the same procedure used for fresh specimens.

Cells. Shell vials and tubes seeded with primary rhesus monkey kidney cells (pRMK) and tubes seeded with human embryonic kidney cells (HEK) were obtained from Viromed Laboratories (Hopkins, Minn.). Tubes and shell vials containing MRC-5 and HEp-2 cells were prepared in-house at least weekly (tubes) or twice weekly (shell vials). HEp-2 shell vials were used within 4 days and MRC-5 shell vials were used within 7 days of planting. All cells were maintained stationary in minimal essential medium (Flow Laboratories, Costa Mesa, Calif.) with 3% fetal calf serum (Sigma Chemical Co., St. Louis, Mo.), 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), and penicillin-streptomycin.

Tube cultures. Specimens were vortexed vigorously, and 0.2-ml aliquots were inoculated into duplicate tubes of pRMK, MRC-5, HEp-2, and HEK cells. Specimens were absorbed on the cell monolayers for 15 min, and the cultures were refed with maintenance medium before being incubated. All tubes were incubated at 37°C, with the exception of one pRMK tube which was incubated at 33°C. The inoculated tubes were examined daily (Monday through Friday) for CPE for a total of 14 days. Tubes were processed for indirect immunofluorescence (see below) when CPE was evident. The 33°C pRMK tubes were processed for indirect immunofluorescence at day 13 or 14, regardless of the presence of CPE at earlier times in the other tubes. Immunofluorescence testing at day 13 or 14 was performed to detect viruses which did not produce CPE and dual infections in which one virus had previously been identified in other tubes by detection of CPE. Tubes were processed for indirect immunofluorescence by rinsing the monolayers with phosphate-buffered saline (PBS) (0.01 M sodium phosphate containing 0.85% NaCl [pH 7.2]) and scraping the cells into a small volume of PBS. The cell suspensions were spotted

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onto the wells of multiwell slides (Cel-Line, Newfield, N.J.), allowed to dry, and fixed with acetone at −20°C.

Shell vials. Single shell vials seeded with pRMK, HEp-2, and MRC-5 cells were inoculated with 0.2-ml specimens after maintenance medium was aspirated. The vials were then centrifuged at 700 × *g* for 40 min at 25°C and refed with 1.0 ml of maintenance medium per vial. The HEp-2 and MRC-5 shell vials were incubated at 37°C and the pRMK shell vials were incubated at 33°C for at least 2 days. Shell vials were not harvested on weekends, so the total incubation period ranged from 2 to 4 days. At the end of the incubation period, the cells in the shell vials were harvested by being scraped into a small volume of PBS, and slides were prepared as described above.

During the first study period (November 1990 to June 1991), three shell vials (pRMK, HEp-2, and MRC-5) were inoculated with each patient specimen. During the second study period (July 1991 to March 1992), only pRMK and HEp-2 shell vials were inoculated.

Indirect immunofluorescence. A panel of monoclonal antibodies (Bartels Diagnostic Division, Issaquah, Wash.) was used for the identification of RSV, adenovirus, influenza A and B viruses, and parainfluenza viruses 1, 2, and 3. Staining was performed as recommended by the manufacturer, and the cells were observed for characteristic fluorescence with a UV microscope (epi-illumination) at magnifications of ×100 and ×400.

The isolation of respiratory viruses in shell vials was attempted with frozen specimens in the first phase of this study to determine whether the procedure could be used successfully for patient specimens. Frozen specimens previously determined to be positive and held at −70°C were thawed and inoculated into shell vials. Ten of 10 specimens known to contain influenza A virus were positive in pRMK shell vials harvested at 2 to 5 days postinoculation. Fifteen of 18 specimens known to contain parainfluenza virus were positive in pRMK shell vials. The three specimens that were negative in shell vials were reinoculated into pRMK tube cultures; one of the three specimens did not grow out parainfluenza virus. Twelve of 15 specimens known to contain adenovirus were positive in shell vials. Two of the three negative specimens contained adenovirus isolated in tube cultures following the inoculation of frozen specimens. Thus, from this small study, it appeared that shell vials could be used successfully for the isolation of respiratory viruses.

During the prospective phase of this evaluation, 359 specimens, including 277 nasal or nasopharyngeal wash specimens, 70 throat swabs and/or washes, 9 endotracheal aspirates, and 3 bronchoalveolar lavage specimens, were processed. A total of 195 respiratory virus isolates were recovered (Table 1). As shown in Table 1, all parainfluenza virus and almost all influenza virus and RSV isolates were recovered in shell vials, but only 10 of 21 adenovirus isolates were detected by the rapid culture technique. The average time to detection of adenovirus specimens that were positive in shell vials was 4 days, compared with 8 days for specimens which were negative in shell vials and positive only in tube cultures. The difference in time to detection of adenovirus shell vial-positive and time to detection of shell vial-negative specimens in tube cultures is statistically significant ($P = 0.0053$ by Student's t test).

Isolation rates of the various viruses in MRC-5, HEp-2, and pRMK shell vials were compared to determine the sensitivity of each individual cell type for detection of the various respiratory viruses (Table 2). pRMK shell vials were optimal for the isolation of influenza viruses, and pRMK and HEp-2 shell vials were equally sensitive for the detection of the parainfluenza viruses. Influenza A and B viruses were occasionally isolated with HEp-2 and MRC-5 shell vials, although the number of positively staining cells was smaller with these cell types than with pRMK. HEp-2 shell vials were optimal for the detection of RSV and adenovirus. MRC-5 shell vials were not used in the second phase of this evaluation, since they conferred no advantage over HEp-2 shell vials.

Since shell vials were not harvested on weekends, the time from inoculation to processing of vials for indirect immunofluorescence varied from 2 to 4 days. The days that shell vials were harvested were compared for specimens that were positive in shells vials and those that were positive only in tube cultures (negative in shell vials). This comparison was performed to determine whether harvesting shell vials at later times would increase the sensitivity for isolation of adenovirus. Adenovirus was detected in one-half of the positive cultures harvested at days 2 and 3 (6 of 12 and 2 of 4, respectively) and 2 of 5 cultures harvested at day 4. Since there was no difference in the percent positive cultures at different harvest times, it would appear that harvesting shell

TABLE 1. Isolation of respiratory viruses from fresh specimens in shell vials and tube cultures

Virus	No. of isolates in[a]:			
	SV + TC	TC only	SV only	SV/total no. isolated
Adenovirus	10	11	0	10/21
Influenza				
A	15	1	0	15/16
B	5	0	0	5/5
Parainfluenza	15	0	0	15/15
RSV	125	11	2	127/138

[a] SV, shell vials; TC, tube cultures.

TABLE 2. Isolation of respiratory viruses in shell vials containing pRMK, MRC-5, or HEp-2 cells

Virus	Condition or study period	No. of isolates in indicated cell type/total no. isolated in shell vials		
		MRC-5	pRMK	HEp-2
Adenovirus	Frozen	4/12	ND[a]	11/12
	1[b]	3/6	2/6	6/6
	2[c]	ND	0/4	4/4
Influenza				
A	2	ND	15/15	1/15
B	1	4/5	5/5	2/5
Parainfluenza				
1	1	7/7	7/7	7/7
2	1	1/2	3/3	3/3
3	1	4/4	5/5	5/5
RSV	1	35/38	34/41	41/41
	2	ND	80/86	84/85

[a] ND, not done.
[b] November 1990 to June 1991.
[c] July 1991 to March 1992.

vials at 3 to 4 days confers no advantage for the detection of adenovirus compared with standard harvesting at 2 days. For RSV, 9% (9 of 96) and 10% (2 of 19) of positive specimens harvested at 2 and 3 days, respectively, were negative in shell vials, whereas all 23 positive specimens harvested at 4 days were positive in shell vials. Although the numbers are small, it would appear from this data that harvesting shell vials at day 4 would identify more RSV isolates than harvesting before this time.

Our results for the detection of RSV in shell vials are very similar to those reported by Matthey et al. (9) and Smith et al. (11). Two other groups have previously reported that shell vials are more sensitive for the detection of RSV than tube cultures, but this may have been due to the use of single tubes (rather than that of duplicate tubes, which enhances recovery) and the processing of only tubes with CPE (7, 10).

Several studies have documented the usefulness of shell vials for the detection of influenza viruses. Three of these studies found that shell vials were not as sensitive as tube cultures for the detection of influenza viruses, with sensitivities ranging from 60 to 91% (2, 4, 12). Two additional reports concluded that pRMK shell vials or MDCK plate cultures were more sensitive than tube cultures for the isolation of influenza viruses (10, 16). In all but one of these studies (10), duplicate shell vials were inoculated and one was stained with monoclonal antibody to influenza A virus and the other was stained with antibody to influenza B virus. Staining two coverslips with monoclonal antibodies can be very expensive, and thus it is advantageous to scrape the cells and spot them onto multiwell slides in order to conserve reagents.

Rabalais et al. (10) found that shell vials were approximately 80% sensitive for the isolation of adenovirus and the parainfluenza viruses. To our knowledge, no other studies which describe the isolation of adenovirus in shell vials primarily from respiratory specimens have been published. The other published reports in the literature describing adenovirus isolation in shell vials either do not elaborate on the source of specimens or use primarily nonrespiratory specimens for comparison. Espy et al. (3) found that HEp-2 shell vials were 52% sensitive at 24 h and 97% sensitive at 48 h postinoculation with specimens known to be previously positive. Woods et al. (17) found that 6 of 13 specimens known to contain adenovirus were positive in A549 24-well plate centrifugation cultures at 24 h and 8 of 13 were positive at 48 h. Mahafzah and Landry (8) compared A549 shell vials with traditional tube cultures for the detection of adenovirus and found that 77% of specimens were positive in shell vials at 2 days and 100% were positive at 5 days postinoculation. The majority of specimens in this study were eye swabs.

The shell vial method used in our study proved very sensitive for detecting all of the respiratory viruses except for adenovirus. The best combination for inoculation with fresh specimens was pRMK and HEp-2 shell vials. MRC-5 shell vials were less sensitive than HEp-2 shell vials for the detection of adenovirus and offered no advantage over the other two shell vials for the detection of the other respiratory viruses. HEp-2 and pRMK shell vials were equally sensitive for the parainfluenza viruses, and HEp-2 shell vials were optimal for RSV, since more isolates were detected in HEp-2 than in the other shell vials and the number of infected cells was usually greatest in the HEp-2 shell vials.

Harvesting of shell vials 4 days after inoculation did not result in increased detection of adenovirus compared with harvesting after 2 days. Thus, it appears necessary to continue the use of tube cultures for the isolation of adenovirus, in conjunction with shell vials. On the basis of the difference in time to detection in tube cultures of specimens positive in shell vials and time to detection in tube cultures of those negative in the shell vials, it appears that shell vials work well for the isolation of adenovirus from specimens containing relatively large amounts of virus but are insensitive for specimens containing small amounts of virus. This was also shown in the study by Mahafzah and Landry (8), who found that detection in shell vials at various days postinoculation was dependent on the amount of virus in the original specimen.

The method used in this study for the isolation of respiratory viruses in shell vials is very cost-effective, requiring only small volumes of reagents for the indirect immunofluorescence procedure and two shell vials. The cost of the shell vial reagents is $8.94, with the majority of this due to the cost of the monoclonal antibody reagents ($6.02 each). If single shell vials were stained, the cost of the shell vial reagents would jump to $33.01. Since the shell vial technique is so sensitive, it would not appear necessary to rely on tube cultures for the detection of any virus other than adenovirus. The number of tube cultures inoculated can be kept to a minimum and examined primarily for CPE produced by adenovirus. This technique can be used efficiently by busy laboratories for the rapid identification of almost all respiratory viruses readily identified by culture.

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