

Detection of Influenza Virus by Centrifugal Inoculation of MDCK Cells and Staining with Monoclonal Antibodies

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Two methods for detection of influenza virus in 451 clinical respiratory specimens were compared: (i) 24-well-plate centrifugation with Madin-Darby canine kidney (MDCK) cells and staining with monoclonal antibody pools to influenza viruses A and B (Centers for Disease Control, Atlanta, Ga.) in an indirect immunofluorescence assay after incubation for 40 h, and (ii) conventional tissue cell culture with primary monkey cells and hemadsorption. For 100 of these specimens, direct examination of smears by the direct fluorescence assay with monoclonal antibodies (Boots Cell Tech/API Analytab Products, Plainview, N.Y.) was also performed. Influenza A virus was recovered from 28 specimens by tissue cell culture after incubation for an average of 4.75 days (range, 2 to 14 days). Influenza B virus was recovered from 35 specimens by tissue culture after incubation for an average of 5.4 days (range, 3 to 14 days). By the centrifugation assay, 23 specimens were positive for influenza A virus and 30 were positive for influenza B virus. All specimens positive by the centrifugation assay were also positive by conventional tissue cell culture. The sensitivities of the centrifugation assay were 82% for detection of influenza A virus and 86% for influenza B virus (84% overall); the specificity of the assay was 100%. Of the 100 specimens studied by direct examination, 15 were positive for influenza virus by both conventional culture and centrifugation assay; however, the direct-smear results for these 15 specimens were negative in 13 cases and inconclusive in 2. The centrifugation assay is a rapid and specific method for detection of influenza A and B viruses in clinical specimens, and it can serve as a valuable and cost-efficient adjunct to conventional culture methods.

During epidemics, the influenza viruses are a significant cause of morbidity and mortality, especially in the elderly and in patients with chronic pulmonary and/or cardiovascular disorders (13). To allow both timely institution of appropriate infection control measures and patient management, rapid detection of influenza virus in clinical specimens is essential. Direct detection of the virus in specimens would be optimal. This has been accomplished by using an immunofluorescence or enzyme-linked immunosorbent assay (EIA) (1, 2, 5, 7, 11). Problems with direct-smear examination by immunofluorescence include low sensitivity and nonspecific background staining, and at present there is no commercially available EIA for direct detection of influenza virus. The shell vial centrifugation assay, initially devised for rapid detection of cytomegalovirus (4), has been adapted for detection of the influenza viruses (2, 12). As an alternative to individual 1-dram (12-mm) vials, 24-well plates have been used successfully in a centrifugation assay for detection of cytomegalovirus (14, 17). Because of the ease of manipulation and cost-effectiveness of using 24-well plates in testing large numbers of specimens, we used the basic technique to detect influenza A and influenza B viruses in clinical respiratory specimens; however, in place of MRC-5 cells, cover slips were seeded with Madin-Darby canine kidney cells, which are very sensitive to infection with influenza virus (3, 8). Smears prepared directly from specimens were also examined by using a direct immunofluorescence assay.

MATERIALS AND METHODS

Specimens. Between January and March 1989, a total of 451 specimens collected from respiratory sites by nursing personnel or respiratory therapists were included in the

study (Table 1). Specimens were not screened to include only patients with influenzalike symptoms. All nasopharyngeal and throat specimens were tested, whereas the centrifugation assay was performed on bronchoalveolar lavage, tracheal aspirate, sputum, lung, adenoid, epiglottis, and vocal cord specimens only if specifically requested. Lung, adenoid, and epiglottis tissue, tracheal aspirates, and sputum specimens were homogenized in Hanks balanced salt solution (Hazelton Dutchland, Inc., Denver, Pa.) by using a sterile mortar and pestle to make a 10 to 20% suspension and then centrifuged at $8,000 \times g$ for 30 min. The cell extract was used for virus recovery. Throat and nasopharyngeal swabs were extracted into 2 ml of Hanks balanced salt solution with gelatin. For bronchoalveolar lavage fluids, if ≥ 5 ml was received, the specimen was centrifuged at $1,000 \times g$ for 10 min, all except 4 ml was removed, and the remaining 4 ml was vortexed prior to inoculation. Bronchoalveolar lavage fluids consisting of < 5 ml and throat wash specimens were not manipulated before inoculation. An antibiotic suspension, consisting of penicillin, gentamicin, and amphotericin B (Fungizone), was added to all specimens prior to inoculation of cell cultures. Specimens were stored at 4°C until further processed (usually within 8 h of receipt).

Plate centrifugation assay. Sterile circular glass cover slips (12-mm diameter) were placed in sterile 24-well plates (Costar, Gaithersburg, Md.) and seeded with Madin-Darby canine kidney cells (MDCK; ATCC CCL 34; American Type Culture Collection, Rockville, Md.) suspended in minimum essential medium (MEM) (Hazelton Dutchland, Inc.) containing penicillin, streptomycin, L-glutamine, 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Irvine Scientific, Santa Ana, Calif.) and 10% fetal calf serum (Hyclone, Logan, Utah). After a confluent monolayer had formed (approximately 2 days after seeding), the medium

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TABLE 1. Comparison of the 24-well-plate centrifugation (24-WPC) assay and conventional culture for detection of influenza virus in clinical specimens

Specimen type (no. tested)	No. of virus-positive specimens detected				Total
	Influenza A virus		Influenza B virus		
	24-WPC	Culture	24-WPC	Culture	
Nasopharyngeal (238)	19	23	18	21	44
Throat (121)	4	5	9	11	16
Bronchoalveolar lavage (64)	0	0	1	1	1
Tracheal aspirate (15)	0	0	1	1	1
Sputum (6)	0	0	1	1	1
Lung (4)	0	0	0	0	0
Other ^a (3)	0	0	0	0	0
Total (451)	23	28	30	35	63

^a Includes one specimen each of adenoid, epiglottis, and lung tissue.

was aspirated, and each of two wells was inoculated with 0.2 ml of specimen. Two wells, one inoculated with known influenza A virus and one with known influenza B virus, served as positive controls. To assess cross-contamination, wells immediately adjacent to the positive control wells were left uninoculated and served as negative controls. Plates were centrifuged at $700 \times g$ for 45 min at 25°C. To each well, 1.0 ml of serum-free MEM supplemented with 2 μ g of trypsin per ml was added, and plates were incubated at 35°C for 40 h. Cover slips were washed twice with phosphate-buffered saline (PBS) and fixed in cold methanol for 10 min. One cover slip of each specimen pair was stained with 100 μ l of a 1:100 dilution of monoclonal antibody pools to influenza A virus and to influenza B virus (Centers for Disease Control, Atlanta, Ga.) (15) and then incubated at 37°C for 30 min. After being washed twice with PBS, cover slips were stained with 100 μ l of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) diluted 1:100 in PBS and incubated at 37°C for 30 min. Monolayers were again washed with PBS, and after counterstaining with 0.05% Evans blue (Fisher Scientific Co., Pittsburgh, Pa.) for 1 min, cover slips were mounted on glass slides and viewed at $\times 100$ and $\times 400$ magnifications with an epifluorescence Leitz microscope. The presence of one or more cells containing positively fluorescing intracytoplasmic and/or intranuclear inclusions was considered positive for influenza virus.

Conventional tube cell culture. The following cell lines were inoculated with 0.2 ml of specimen: primary rhesus monkey kidney cells (PMK) (Bartels Immunodiagnostic Supplies, Bellview, Calif.), HEp-2 cells (ATCC CCL 23), A549 cells (ATCC CCL 185), and MRC-5 cells (Bartels Immunodiagnostic Supplies). Specimens were adsorbed for 1 h at 35 to 36°C, 1 ml of MEM was added to each tube (serum-free MEM for PMK), and tubes were incubated at 33 to 34°C. Cultures were examined three times per week for 3 weeks for virus-specific cytopathic effect (CPE). If no CPE had developed in the PMK cells by days 4 to 6, hemadsorption with a 0.4% suspension of guinea pig erythrocytes was performed. Hemadsorption was repeated on days 10 to 14 if the initial test was negative. Cell monolayers from hemadsorption-positive cultures were washed with PBS, and the cells were removed by gentle scraping, placed on glass slides, air dried, fixed with acetone, and stained by indirect immunofluorescence with monoclonal antibodies to influenza A and B viruses (Centers for Disease Control) and with

a pan-parainfluenza virus monoclonal antibody (Chemicon International Inc., El Segundo, Calif.). When CPE was recognized, isolates were confirmed by direct immunofluorescence with monoclonal antibodies (herpes simplex virus, Syva Co., Palo Alto, Calif.; respiratory syncytial virus, Ortho Diagnostics, Inc., Raritan, N.J.) or by indirect immunofluorescence with monoclonal antibodies (adenovirus, Chemicon International Inc.; influenza virus, Centers for Disease Control; cytomegalovirus, Du Pont Co., Inc., Billerica, Mass.). Enterovirus and rhinovirus were identified by appearance of CPE, growth characteristics, and acid sensitivity (6).

Examination of direct smears. Direct smears of 100 acceptable specimens were examined. Each specimen (minimum of 0.5 ml) was placed in a conical tube (14 mm) and centrifuged at $500 \times g$ for 10 min. The supernatant was discarded, and the cell pellet was washed three times in 5 ml of PBS. After the last wash, the PBS was discarded, and the cell pellet was resuspended in 2 drops of PBS. A wet preparation of the specimen was examined; acellular or minimally cellular specimens were considered unacceptable and were not processed further. Two smears per acceptable specimen were prepared on glass slides and allowed to air dry. One slide was stained with 25 μ l of fluorescein isothiocyanate-labeled monoclonal antibody to influenza A virus, and one was stained with monoclonal antibody to influenza B virus (Boots Cell Tech/API Analytab Products). Slides were incubated for 15 min at 37°C in a moist chamber, washed in PBS for 5 min, air dried, cover-slipped, and then viewed with an epifluorescence microscope. Slides of known influenza A and B virus isolates were stained with each specimen run.

RESULTS

Influenza virus was detected in 63 specimens (14%) (28 influenza A virus, 35 influenza B virus). Results obtained by conventional culture and the 24-well-plate centrifugation assay are shown in Table 1. By the centrifugation assay, 23 specimens were positive for influenza A virus and 30 were positive for influenza B virus. All specimens positive for influenza virus by the centrifugation assay were also positive by conventional tissue cell culture. In eight cases the results of the centrifugation assay were inconclusive due to tearing of the cell monolayer. Influenza B virus was recovered by conventional culture from one of these eight specimens; cultures of the remaining seven were negative.

Of the 28 isolates of influenza A virus detected by conventional culture, CPE developed in 20 after incubation for 2 to 6 days, and 8 were positive by hemadsorption at days 3 to 14. The centrifugation assay was positive in 18 of the 20 positive by CPE and in five of eight positive by hemadsorption. For three of the eight hemadsorption-positive specimens, the initial hemadsorption test (day 5) was negative. The second hemadsorption assay (day 10 or 11) was positive for one of the three specimens; the remaining two were hemadsorption positive at day 14. One of the latter two specimens was positive by centrifugation assay. The mean time to detection of influenza A virus by conventional culture in specimens that were also positive by centrifugation assay was 4.5 days (range, 2 to 14 days). For specimens negative by the centrifugation assay, influenza A virus was detected after incubation for a mean of 8.2 days (range, 4 to 14 days).

CPE developed in 26 specimens positive for influenza B virus after incubation for 3 to 6 days. Twenty-five of these were positive by the centrifugation assay, and due to tearing

of the cell sheet, the result of the assay for the remaining specimen was inconclusive. Influenza B virus was detected in nine specimens by hemadsorption at days 4 to 14. Of the nine, five were positive by the centrifugation assay. For three hemadsorption-positive specimens, only the second hemadsorption assay (days 10 to 14) was positive. One of the three was also positive by the centrifugation assay. The mean time to detection of influenza B virus by conventional culture in specimens that were also positive by centrifugation assay was 5.1 days (range, 3 to 12 days). For specimens that were negative by the centrifugation assay, influenza B virus was detected after incubation for a mean of 7.4 days (range, 3 to 14 days).

The sensitivities of centrifugal inoculation of MDCK cells followed by staining with monoclonal antibodies after incubation for 40 h for detection of influenza virus in clinical specimens were 82% for influenza A virus and 86% for influenza B virus (84% overall). The centrifugation assay was 100% specific.

Of the 100 smears examined by the direct immunofluorescence assay, influenza virus was detected in 15 (4 influenza A virus and 11 influenza B virus) by both the centrifugation assay and conventional culture. The direct-smear results were negative for 13 of the 15 specimens and inconclusive due to the high degree of background staining for the remaining 2 (both influenza B virus). The 85 specimens negative for influenza virus by conventional culture were also negative by examination of direct smears.

Of the 388 influenza virus-negative specimens, a virus other than influenza virus was detected in 53: 20 respiratory syncytial virus, 12 parainfluenza virus, 7 rhinovirus, 4 cytomegalovirus, 3 herpes simplex virus, and 3 adenovirus.

DISCUSSION

Rapid diagnosis of infection with influenza virus is important for infection control and therapeutic reasons. The optimal system would allow detection of virus within a few hours after receipt of the specimen. EIA methods have been used (5, 7), but none are commercially available. Examination of direct specimen smears by either indirect or direct immunofluorescence with monoclonal antibodies is insensitive (1, 11, 12), and the slides are often difficult to interpret. Our findings substantiate the insensitivity of direct-smear examination and reiterate the interpretation problems.

Alternatives to EIA and direct-smear examination include centrifugation assays (2, 12), slide chamber assay (12), immunoperoxidase staining of uncentrifuged PMK monolayers (13), and early hemadsorption (9). Espy and colleagues (2) used shell vials seeded with rhesus monkey kidney cells and, following centrifugation and incubation, stained with monoclonal antibody pools in an indirect immunofluorescence assay. After overnight incubation, the sensitivity of their assay compared with tissue culture was 56%, and this increased to 60% after incubation for 48 h. Moreover, they found that centrifugation of the inoculated shell vials increased the number of fluorescent foci observed. Stokes and colleagues (12) evaluated both a shell vial centrifugation assay and a chamber slide assay (without centrifugation) with MDCK cells and monoclonal antibody pools in an indirect immunofluorescence assay. Compared with culture, the sensitivities of shell vial centrifugation and the chamber slide assay after overnight incubation were 84 and 75%, respectively. Swenson and Kaplan (13) tested known influenza virus-positive specimens that had been stored at -70°C by indirect immunoperoxidase staining of uncentrifuged

rhesus monkey kidney cell monolayer 24 h postinoculation. Compared with culture, immunoperoxidase staining of uncentrifuged specimens with monoclonal antibodies had a sensitivity of 86%.

The 84% overall sensitivity of our 24-well-plate centrifugation assay with MDCK cells followed by staining with monoclonal antibodies after incubation for 40 h was virtually identical to that of an overnight shell vial centrifugation assay with MDCK cell-seeded cover slips (11). Our decision to incubate for 40 h was based on the results of a previous evaluation of influenza virus detection with the 24-well-plate centrifugation assay. In that study, the sensitivities of the assay were 76% at 18 h and 91% at 40 h (16). The majority of specimens not detected at 18 h were received from outside hospitals and had been refrigerated for more than 24 h before processing. Because many of our specimens do come from outside hospitals and therefore may undergo prolonged refrigeration, we believed that incubation for 40 h would ensure optimal detection most cost-effectively.

By inoculating one tube each of PMK and cynomolgus monkey kidney cells and testing alternate tubes for hemadsorption on days 1 to 5 after inoculation, Minnich and Ray (9) were able to detect all influenza A virus isolates by day 3 and all influenza B virus isolates by day 4. Although our study was not designed to repeat these results, we would not have detected at least six isolates if we had followed that protocol. One isolate of influenza A virus was hemadsorption negative at day 5 but positive at day 10, and two influenza A virus isolates were hemadsorption negative both at day 5 and at day 10 or 11, but positive at day 14. Three isolates of influenza B virus were hemadsorption negative at day 5 or 6 but positive at day 10, 12, or 14.

In conclusion, the 24-well-plate centrifugation assay provides rapid and specific detection of influenza virus. The potential advantages of using 24-well plates rather than shell vials include ease of manipulation and increased cost efficiency when processing multiple specimens. Given that the assay had an overall sensitivity of 84%, we do not believe that it should serve as a culture substitute. Rather, the assay should be used in conjunction with culture and hemadsorption. Importantly, the combination of centrifugation assay plus one tube of PMK cells is as cost-effective as inoculating two tubes of PMK cells and performing serial hemadsorptions. Moreover, since the majority of positive specimens will be detected by the centrifugation assay, only one hemadsorption assay at days 10 to 14 should be necessary to detect the remaining influenza viruses and all parainfluenza virus isolates (9). Direct-smear examination, on the other hand, was not helpful in the rapid detection of influenza virus.

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