Early Detection of Influenza Virus by Using a Fluorometric Assay of Infected Tissue Culture

CONSTANCE T. PACHUCKI* AND CATHERINE CRETICOS†

Section of Infectious Disease (111P), Edward Hines, Jr., Veterans Administration Hospital, Hines, Illinois 60141,* and Stritch School of Medicine, Loyola University, Maywood, Illinois 60153

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A fluorometric substrate, 4-methylumbelliferyl-α-ketoside of N-acetylneuramidase, was used directly on clinical specimens and infected tissue culture 24 h after inoculation for the detection of influenza viral neuraminidase. Viral neuraminidase was detected in infected tissue culture but not in clinical specimens. The sensitivity of the assay on tissue culture was 92%, and the specificity was 96%.

Early detection of influenza virus in infected tissue culture is important for the confirmation of the clinical diagnosis of influenza and the subsequent initiation of antiviral chemotheraphy and other preventive measures (2, 11). A fluorometric substrate, 4-methylumbelliferyl-α-ketoside of N-acetylneuramidase, was used on clinical specimens and infected tissue culture to detect influenza viral neuraminidase 18 to 24 h after inoculation. The neuraminidase activity of surface glycoproteins of both influenza viruses A and B hydrolyzes the α-ketoside bond of N-acetylneuraminic acid to produce an intensely fluorescent product (6). EDTA selectively inhibits the fluorescence generated from viral neuraminidase which distinguishes it from bacterial or human neuraminidases (3). Prior work showed that this substrate detects 100% 50% tissue culture infective dose of influenza viruses A and B and 75% of virus-positive specimens obtained from experimentally infected volunteers (12). This fluorometric substrate had not been used on clinical specimens collected from patients with upper respiratory illness. The results of such application are described here.

(This paper was presented in part previously [C. T. Pachucki and L. Braune, Program Abstr. 26th Internsci. Conf. Antimicrob. Agents Chemother., abstr. no. 93, 1986].)

Throat-wash specimens were collected in 10-ml sterile normal saline from employees and patients at Edward Hines, Jr., Veterans Administration Hospital with upper respiratory illness during three consecutive winters. Throat-wash specimens were placed on triplicate tubes of primary Rhesus monkey kidney (PRMK), HEP-2, and WI-38 human lung fibroblast tissue cultures. Respiratory viruses were identified by using standard techniques (1, 4, 7). Aliquots of specimens were frozen at −70°C in a Revco freezer for later testing with the fluorometric assay.

Influenza virus A/Philippines H3N2 stock culture and purified viral neuraminidase (California Biochemical Corp., Palo Alto, Calif.) were used as positive controls (see Fig. 1 and 2). Throat-wash specimens from healthy individuals; supernatant and freeze-thawed tissue culture from uninfected PRMK tissue culture, undiluted and diluted; phosphate-buffered saline; and dilutions of Streptococcus viridans cultures were used as negative controls. Infected PRMK tissue culture was hemadsorbed 18 to 24 h after inoculation. Supernatant was pooled with freeze-thawed tissue culture and assayed for neuraminidase. Duplicate tubes of PRMK were incubated for 7 to 10 days for identification of influenza viruses.

Triplicate 0.1-ml samples of specimen were incubated with 0.1 ml of the fluorescent substrate (4-methylumbelliferyl-α-ketoside of N-acetylneuraminidase; Koch-Light Laboratories, Ltd., Haverhill, Suffolk, England) diluted to a concentration of 30 μg/ml (10−4 M) in 0.1 M phosphate buffer (pH 5.8) containing 0.001 M CaCl2. The mixture was incubated in borosilicate tubes at 37°C for 90 min. The volume of the tubes was brought to 1.0 ml by the addition of 0.01 M Tris buffer (pH 8.2), and the fluorescence was measured in a fluorometer at an incident transmission wavelength of 360 nm with a 440-nm interference filter with a 10-nm bandwidth (Ferrand Optical). Triplicate tubes were incubated in the same manner with 0.1 M phosphate buffer (pH 5.8) containing 0.01 M EDTA (12).

The relative efficiencies of the fluorometric assay were determined by using viral isolation as the standard. The sensitivity, specificity, and positive and negative predictive values were calculated as previously described (8).

A total of 178 throat-wash specimens were collected from patients with febrile upper respiratory illness; 68 were assayed directly for viral neuraminidase, and 110 were assayed 18 to 24 h after inoculation onto PRMK tissue culture. Specific fluorescences of clinical specimens in which influenza virus was isolated were 2.3 to 12.8 (median, 9.0). These specimens were inhibited 20 to 47% by EDTA. Clinical specimens, which had no influenza viral isolation, had fluorescence values of 0.5 to 68.6 (median, 6.3) and were inhibited 10 to 49% by EDTA. A sample of 10 clinical specimens were filtered through a 0.45-μm (pore size) Millipore membrane before and after the fluorometric assay, with no difference in fluorescence. Specific fluorescence of 10 throat-gargle specimens from healthy adults ranged from 2.7 to 17.3 (median, 5.3) and was inhibited from 20 to 44% by EDTA. The assay reliably detected 0.02 U of purified viral neuraminidase and greater than 100-2 50% tissue culture infective dose of influenza virus A/Philippines H3N2 (Fig. 1 and 2). EDTA inhibited purified viral neuraminidase 23 to 60% and inhibited influenza virus A 61%. Dilutions of S. viridans fluoresced but were not inhibited by EDTA. By using this information, a fluorescence value of 7.5 with ≥20% inhibition with EDTA defined viral neuraminidase in clinical specimens. The results are shown in Table 1. The calculated sensitivity of the test done on clinical specimens was 69%, and the specificity was 56%.

* Corresponding author.
† Present address: Illinois Masonic Medical Center, Chicago, IL 60657.

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FIG. 1. Specific fluorescence activity of influenza virus A with and without EDTA. Standard deviations of points not noted were too small to accurately show on the figure. Open triangle corresponds to the negative control for fluorescence values for influenza virus A H3N2-EDTA. Closed triangle corresponds to the negative control for fluorescence values for influenza virus A H1N1-EDTA. TCID50, 90% Tissue culture infective dose.

The remaining 110 specimens were assayed after incubation in tissue culture. In infected tissue culture, a mean value of unknown specimens which was ±2 standard deviations above control values of uninfected tissue culture and inhibited >30% with EDTA was positive for viral neuraminidase (12). The results are shown in Table 2. The calculated sensitivity of the assay was 92%, the specificity was 96%, the positive predictive value was 73%, and the negative predictive value was 99%.

The fluorometric assay was used on clinical specimens and infected tissue culture in an attempt to develop it for use in a clinical laboratory for the rapid diagnosis of influenza. The assay was not useful in detecting neuraminidase directly in clinical specimens but identified 91% of virus-positive isolates 24 h after inoculation onto tissue culture. This is superior to other early-detection methods, hemadsorption, enzyme immunoassay, and a time-resolved fluoroimmunoassay, which, respectively, detected 38 and 12% of influenza virus at 12 h and 80% at 48 h (5, 9, 10).

As applied, the assay can identify viral neuraminidase from both influenza and parainfluenza viruses (12). To identify the type of viral neuraminidase, routine techniques, such as hemadsorption inhibition (1), indirect immunofluorescence (9), or enzyme immunoassay (12), would have to be performed 3 to 10 days after inoculation of the tissue culture. During epidemic months for patients with a clinical illness compatible with influenza, this assay could provide very early collaborative data for the diagnosis of influenza.

Viral neuraminidase in throat-wash specimens could not be identified because of both inadequate sensitivity and specificity of the substrate. In controls, EDTA significantly inhibited virus seed preparations, whereas bacterial neuraminidase was not inhibited. However, throat washes from uninfected adults were inhibited 20 to 44% by EDTA, suggesting that the fluorescence was nonspecific and related to contaminating neuraminidase and could not be differentiated by inhibition with EDTA. To remove bacteria and other particulate matter, the specimens were filtered before assay; however, the fluorescence values were the same. Specimens were collected 24 to 72 h after the onset of illness in 10 ml of normal saline, so that virus titers of specimens varied and were diluted. In addition, specimens were frozen and thawed once, which may have decreased sensitivity by up to 20% with each cycle (12). At best, the fluorometric substrate detected lower titers of influenza virus stock cultures than had been previously reported (12). The fluorescent substrate used here was obtained from a commercial source, and variable sensitivity of the fluorometric substrate among strains of influenza viruses has been reported (12), both of which might explain this lowered sensitivity. Low virus titers, lower sensitivity of the fluorescent substrate, and the presence of confounding bacterial and human neuraminidases were all responsible for the unsuccessful application of this substrate to identify influenza virus in throat-gargoyle specimens collected from ill patients.

TABLE 1. Detection of viral neuraminidase in clinical specimens comparing the fluorometric assay with viral isolation

<table>
<thead>
<tr>
<th>Virus isolated</th>
<th>No. of fluorometric assay results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Influenza virus A H3N2</td>
<td>11</td>
</tr>
<tr>
<td>Herpes simplex virus</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>23</td>
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</table>
TABLE 2. Detection of viral neuraminidase 24 h after inoculation of PRMK tissue culture compared with viral isolation

<table>
<thead>
<tr>
<th>Virus isolated</th>
<th>No. of fluorometric assay results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Influenza virus A/Philippines H3N2</td>
<td>11a</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>0</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
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</table>

a Of the 12 influenza virus isolates, 2 hemadsorbed at 24 h.

The use of this fluorometric assay on infected tissue culture provides a specific, sensitive method for the early detection of viral neuraminidase. It has an easily identifiable endpoint, detects all influenza viruses without regard to antigenic changes, and utilizes equipment which may be available in a clinical laboratory.

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LITERATURE CITED


