Noninstrumental Detection of Influenza Viruses by Enzyme Immunoassay

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Introduction

Influenza viruses are usually associated with epidemic respiratory infections in all age groups (Glenzen 1982; Kendal and Walls 1987). Influenza A and B produce symptoms in humans that are not pathognomonic. During epidemics, the infection may be diagnosed on a clinical basis. However, isolated cases, as well as patients seen at the beginning of an outbreak between epidemics, are difficult to diagnose clinically. A series of infectious agents such as respiratory syncytial virus, adenovirus, corona virus, rhinovirus, the parainfluenza virus group, and *Mycoplasma pneumonia* can produce the clinical symptoms often designated as "the flu" (Huley 1987). Definitive diagnosis of influenza virus infection has traditionally relied on viral isolation or serology. However, the time required for laboratory diagnosis has often exceeded the duration of the disease. These delayed laboratory reports coupled with the lack of specific therapy resulted in decreased interest in obtaining a laboratory diagnosis. However, the recent availability of specific antiviral agents and rapid diagnostic techniques have revived the interest in laboratory diagnosis.

Prevention and treatment of infections from influenza A and B are very important because of the frequency and potential severity of the infection. Many studies have demonstrated that amantadine and rimantadine are effective prophylactic and therapeutic agents against infection caused by various strains of influenza A virus (Wingfield et al. 1969; Barker and Mullooly 1980). Since these two drugs are ineffective against influenza B and other viral respiratory infections, such as RSV and parainfluenza types, it is essential to have a positive diagnosis before therapy. In addition, an easy to use and accurate laboratory diagnosis of influenza A in the field would enable accurate tracking of the epidemic.

In this report we present the rapid detection of influenza antigens using an immunofiltration format. The assay can be performed in 25 min and can discriminate readily between influenza A and B using visual comparative detection of colored tabs (noninstrumental).

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Materials and Methods

Immunofiltration Format

Enzyme immunoassay is used in most virology laboratories for the quantitative estimation of antigens. The standard format is a solid phase sensitized with one or more antibodies and a second antibody directed against a different antigenic epitope that is labeled with a reporter enzyme. If the antigen is present in the clinical sample, the antigen is sandwiched between the solid-phase and liquid-phase antibodies. Unbound antibody-enzyme conjugates are removed by washing and the antigen can be quantitated indirectly by quantitating the bound enzyme activity using appropriate substrate. Several variations of this basic scheme have been put into practice. The most commonly used solid phases are microtiter plates or polystyrene beads. The disadvantages of these formats include the slow kinetics of immunologic reactions and the laborious washing steps (the need for automated washers). Of crucial importance to influenza viruses is the slow kinetics of standard ELISA formats due to small sensitizable surface area. Much faster and efficient reaction kinetics can be achieved using microparticles such as the solid phase. Latex particles averaging 0.8 μm in diameter were coated with anti-influenza A antibodies (designated A1) by incubating washed latex particles with 100 μg/ml of protein A column purified monoclonal antibody. A second monoclonal antibody (designated A3) was conjugated with alkaline phosphatase. Virus isolates or clinical samples were diluted in a specimen diluent and mixed with antibody-coated latex particles and antibody-enzyme conjugate as shown in Fig. 1 for 10 min at room temperature. The mixture is then transfered to the filtration device. Latex particles are collected on the membrane. The specific viral antigens present in the sample can bridge the solid-phase antibody to the antibody-enzyme conjugate. Unbound antibodies pass through the filter. Latex-bound conjugates are then visualized by reacting with a precipitable substrate (BCIP-NBT). Viral antigen positive

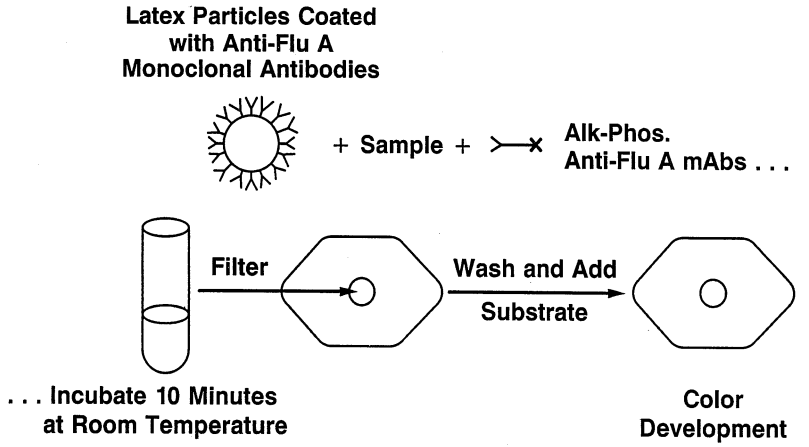

Fig. 1. Flu-A antigen test

samples give deep blue color while negatives are colorless. The reaction can be scored after 10-min incubation at room temperature or made permanent by stopping the reaction with 1N H_2SO_4.

Influenza B test was developed with an identical format using anti-influenza B monoclonal antibodies. Microparticles were sensitized with monoclonal antibody (designated B1) and alkaline phosphatase was conjugated to monoclonal antibody B3. Specificities of the monoclonal antibodies used in the study are described elsewhere (Walls et al. 1986a).

Clinical Samples

Tissue Culture Isolates. A total of 154 isolates from two different medical centers were used in this study. Fourty-five isolates were obtained from Nassau Medical Center that were identified as influenza A virus. Of 109 isolates obtained from Columbia Presbyterian Hospital 53 were identified as influenza A and 56 as influenza B. These viruses were isolated from nasal swabs from patients presenting influenza-like symptoms by inoculating tissue cultures of cells.

Direct Detection from Nasopharyngeal Swabs. In a nonepidemic year (1988). 108 clinical samples in the form of nasopharyngeal swabs were collected at Hackensak Medical Center, Hackensack, NJ. Of these 103 were culture negative for influenza A and B, 3 were RSV positive, and 1 was influenza A positive.

In addition, 86 nasopharyngeal swabs with known culture results were obtained from Columbia Pres byterian Hospital. Of these 63 were culture negative, 7 Flu A positive, 2 parainfluenza positive, 2 rhinovirus positive, and 2 herpes virus positive. (These specimens were tested for Flu-A antigen by immunofiltration format.) Also, 25 influenza B culture-positive, 8 influenza A-positive specimens were obtained from the University of Michigan, Ann Arbor, Michigan, and 6 culture-confirmed influenza A specimens were obtained from Washington University, St. Louis, Missouri.

Assay Procedure

One hundred μ l of clinical specimen (nasal/throat swab in transport medium), 100 μl of specimen diluent, 5 μl of 2% (wt/vol) latex particles sensitized with antibody, and 200 μl of antibody -alkaline phosphatase conjugate are mixed and incubated for 10 min at room temperature. The mixture is then added dropwise to the filtration device. Add 200 μl (x2) wash solution to the device and 100 μl of freshly prepared BCIP-NBT substrate solution. Incubate for 10 min at room temperature for color development. The reaction can be scored immediately or stopped for future scoring by the addition of 100 μl of 1N H_2SO_4. If the reaction is not stopped, negative samples develop color slowly (within 1-2 h). The procedure is graphically represented in Fig. 1. Purple-blue color represents a positive reaction, while pale yellow represents a negative reaction.

Controls

A dilution series of influenza-A H1N1 isolates at various pfu levels ranging from 1×10^2 to 1×10^6 were used with each run. The low positive control was 1×10^2 to determine visual cut off. Tissue culture media and pooled negative samples were also run as negative control. Samples were scored "weakly reactive" when visual color intensity was equal to the low positive control. Low positive control was always clearly distinguishable from negative controls.

Positive control series for influenza B were prepared from tissue culture isolates of influenza B. Common negatives were used for both Flu-A and -B tests.

Analytical Sensitivity

In order to determine the analytical sensitivity of the test, the H1N1 isolate at 6×10^8 pfu/ml stock solution was spiked into tissue culture media at tenfold dilutions and an immunofiltration assay was performed on both Flu-A and -B assays. Clear positive signals were generated at the 1×10^2 pfu/test level.

Results

When 45 Flu-A isolates (H1N1) samples from Nassau Medical Center were analyzed by rapid filtration assay, 44 out of 45 showed positive reactions (39 very strong, 4 a weak reaction at 10-min incubation time). One of the positive isolates showed a negative reaction (Table 1).

When 53 Flu-A isolates from Columbia Presbyterian Hospital were tested by the filtration method, all 53 showed positive reactions (41 strong and 12 weak reactions; Table 2). All 56 Flu-B isolates from Columbian Presbyterian Hospital also showed a specific positive reaction when tested with the Flu-B filtration assay. In this study 51 showed a strong reaction, while 5 showed weak but clearly positive (Table 3) reactions. There was no visible reaction when Flu-A samples were tested with the Flu-B test or when Flu-B samples were tested with Flu-A assay. Out of 108 clinical samples, 3 showed reactivity using the Flu-A assay. Only one out of

Table 1. Influenza culture isolates detection study (Nassau Medical Center)

Flu A-H1N1		
Total Number of Isolates:	45	
EIA Positive:	44	
	39	Showed Strong Reaction
	5	Showed Weak Reaction
EIA Negative:	1	
	+	–
Culture	45	0
Roche EIA	44	1

Table 2. Influenza Culture Isolates Detection Study (Isolates from Columbia Presbyterian Hospital)

Flu A		
Total Number Tested:	53	
EIA Positive:	53	
	41	Showed Strong Reaction
	12	Showed Weak Reaction
	+	–
Culture	53	0
Roche EIA	53	1

Table 3. Flu-B Isolates

Total Number Tested:	56	
EIA Positive:	56	
	51	Showed Strong Reaction
	5	Showed Weak Reaction
	+	–
Culture	56	0
Roche EIA	56	1

these three were culture positive. None of the three showed reactivity with the Flu-B test. Out of 105 negative samples, 16 showed a higher background color than expected. However, this background noise was also evident with the Flu-B assay, indicating possible filtration problems with these samples. Further development on the filtration device system eliminated background problems (Table 4).

When 25 Flu-B clinical samples were tested with Flu-B assay, 21 showed strong reactivity, 3 weak reactivity, and 1 showed a negative reaction (Table 5).

Out of 14 Flu-A samples from the University of Michigan and Washington University, St. Louis, 10 showed strong reactivity and while clearly positive 3 showed weaker signals with the Flu-A test. One culture-positive sample was negative by this assay (Table 6).

Out of 86 clinical samples obtained from Columbia Presbyterian Hospital, the immunofiltration assay showed 8 strong positive reactions (7 culture-positive and 1 culture-negative); 80 samples showed a negative reaction. However, ten of these samples showed higher backgroud signals than expected (Table 7). Although the sample size is not large enough, current sensitivity of this test with Flu-A clinical samples is 95% (21/22). However, only 81% showed strong reactivity.

Flu-A and -B isolates when tested with Roche Filtration EIA gave more than 99% sensitivity and specificity (Tables 1-3) compared to tissue culture. In the samples collected during a nonepidemic year (Hackensack Study), the specificity with clinical samples was 98% (105/107 Flu negatives). Sensitivity could not be determined from this study due to the very low incidence of Flu positives (1/108).

Table 4. Clinical Specimens (Hackensack Medical Center

Total Number	Flu A Culture Positive	RSV Culture Positive	Questionable Culture	Culture Negative
108	1	3	1	103
EIA Screening – Flu A				
EIA Positive	EIA Negative with Background		EIA Negative without Any Background	
3	16		89	

Table 5. Flu-B culture positive clinical specimens (University of Michigan)

Total Number: 25		
	+	–
Culture	25	0
Roche EIA	24	1

Table 6. Flu-A clinical specimen

Total Number Tested:	22	
EIA Positive	21	
	18	Showed Strong Reaction
	3	Showed Weak Reaction
	+	–
Culture	22	0
Roche EIA	21	1

Table 7. Clinical specimens (Columbia Presbyterian Hospital)

Total Number	Culture Negative	Flu A Culture Positive	RSV Culture Positive	Para-Flu	Rhino	Herpes
89	63	7	13	2	2	2
Roche EIA Screening with *Flu A Antibody*						
EIA Positive		EIA Negative with Background		EIA Negative without Any Background		
8		10		71		

In the Michigan study, using 25 confirmed Flu-B positive clinical samples, the sensitivity was 96%. It should be noted that only 84% of these samples showed a strong reaction.

Discussion

Various approaches to the rapid diagnosis of influenza viral antigens in respiratory secretions and tissue culture have been described. These include fluorescence microscopy of stained cells (McQuillin 1985; Epsy et al. 1986), enzyme immunoassays with colorimetric (Harmon and Pawlik 1982) or fluorogenic substrate (Berg et al. 1980), radioisotopic immunoassays (Sarkkinen et al. 1981), and time-resolved fluoroimmunoassays (Walls et al. 1986b). Some of these investigations (Epsy et al. 1986; Walls et al. 1986a, b) utilized the same monoclonal antibodies used in this study (A1, A3, B1, B4). It is clear from these studies that the observed specificity and sensitivity of these monoclonal antibody preparations were dependent on the technique used for detection. For example, using the same monoclonal antibodies, the observed sensitivity for Flu A ranged from 69% for fluorescence staining (Shalit et al. 1985) to 85% for time-resolved fluoroimmunoassay. Specificities of these two assays were also different (86% versus 95%) between these formats. Background signals generated by interfering substances present in nasopharyngeal specimens could also influence the sensitivity and specificity of the assay in a format-dependent manner.

Enzyme immunoassays have evolved over the last decade from complicated multistep procedures to simple tests producing results in minutes. Filtration formats and the availability of diverse solid phases have contributed to these advances. Use of micro-particles as the solid phase for antibody binding results in enhanced kinetics of the antigen-antibody reaction approaching liquid kinetics. Filtration of these particles through synthetic membranes with low protein-binding characteristics allows the visualization of the enzymatic reaction with minimal background problems. Development of passive filtration devices has allowed noninstrumental manipulation of EIA steps. Commercial availability of high-capacity adsorbents have also contributed to these disposable ready-to-use immunofiltration devices. We chose to use latex particles as immunosorbant solid phase and Loprodyne (Pall Corp, NY). Under the described experimental conditions, the filtration immunoassay gave visually discriminable signal-to-noise separation.

Using the current format, the observed sensitivity and specificity on flu isolates were excellent (>99%). Specificity on nasal swabs and throat swab specimens was also at an acceptable level (98%). Although the analytical sensitivity of the test showed clear positive reactions up to 100 pfu of Flu A spiked into negative pool samples, only 82% of the stored clinical samples (39 out of 47 Flu-A and -B samples tested) showed strong reactivity. This could be due to either a titer below 100 pfu in these samples or insufficient release of viral antigens from clinical samples. Variation in sensitivity for Flu-A and -B tests between culture isolates and clinical samples could also be due to a large excess of defective

virus/free antigens that may be present under culture conditions. Appropriate modifications of specimen diluent and incubation times should improve the clinical sensitivity.

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