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# Time-Resolved Fluoroimmunoassay with Monoclonal Antibodies for Rapid Diagnosis of Influenza Infections

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**Monoclonal antibodies that are broadly reactive with either influenza A or influenza B viruses were used to develop a 2- to 3-h antigen capture time-resolved fluoroimmunoassay (TR FIA) for detecting influenza viral antigens in both original nasopharyngeal aspirate specimens and in tissue cultures inoculated with nose or throat swab specimens. The lower limit of sensitivity of the assay was about 10 pg of protein as determined with purified influenza A nucleoprotein expressed by recombinant DNA. When the TR FIA was performed with 96 nasopharyngeal aspirate specimens collected during outbreaks of influenza A (H3N2) virus and the results were compared with serodiagnosis results with paired sera, the specificity and sensitivity of TR FIA for the demonstration of influenza A infections were 95 and 85%, respectively. In culture confirmation assays, more than 80% of the swab specimens that grew influenza A or B virus within 7 days could be identified by the TR FIA within 48 h of the inoculation of cells. The results are consistent with those previously reported for respiratory syncytical virus and extend the applicability of monoclonal antibody-based TR FIA for the rapid diagnosis of acute respiratory viral infections.**

As effective antiviral drugs for preventing and treating influenza become more available, there is an increased need for practical diagnostic tests that can be used either in managing of individual patients or in community-wide control activities, particularly for influenza (2). Various approaches to the rapid diagnosis of influenza virus infections involving antigen detection have been described. Influenza viral antigens in respiratory secretions have been detected by fluorescence microscopy of stained cells (9), enzyme immunoassays with colorimetric (5, 11) or fluorogenic (1, 6) substrates, and radioisotopic immunoassays (11). In addition, virus-specific neuraminidase activity has been measured with fluorescent substrates (16). While each approach has certain advantages and disadvantages, a common problem hindering the routine use of any procedure has been the lack of consistent, high-quality immunoreagents. To address this issue and provide the foundation for diagnostic test development, monoclonal antibodies that are broadly reactive with either influenza A or influenza B viruses have been developed in our laboratories (15). The antibodies have been shown to be useful reagents for directly detecting influenza viruses in nasopharyngeal aspirate specimens (NPS) by the indirect fluorescent antibody technique (9, 13) and for culture confirmation by enzyme immunoassay (EIA) or indirect fluorescent antibody tests of tissue cultures inoculated with throat swab specimens (15).

Recently, a new conceptual and technological approach for quantitating antigen-antibody reactions has been described. Time-resolved fluoroimmunoassay (TR FIA) procedures have provided a rapid and sensitive alternative to EIA or radioimmunoassay (RIA) for objective, quantitative detection of viral antigens in original specimens (4). We therefore examined the feasibility of using monoclonal antibodies in TR FIA for influenza diagnosis, thus extending the advantages of reproducible reagents to this test system also.

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## MATERIALS AND METHODS

**Clinical specimens and reference influenza viruses.** Frozen (−20°C) NPS were collected at the University of Turku in Turku, Finland, and had been previously tested by capture EIA (11). The specimens were prepared as described previously (3) by sonication for at least 2 min after dilution in phosphate-buffered saline (pH 7.3)–2% Tween 20–0.004% thimerosal (specimen diluent). Although not necessary for TR FIA, 20% fetal calf serum was included in the specimen diluent because it was required for EIA tests (11). A separate group of NPS was collected from patients who sought medical attention for respiratory illness during an influenza A (H3N2) epidemic. Paired serum specimens were also collected from each of these patients. The sera were tested for immunoglobulin G (IgG) and IgM antibodies to influenza A by EIA by using partially purified whole virus antigen in an assay similar to a previously described procedure (10). Most sera were also tested by hemagglutination inhibition (8) for antibodies to influenza A (H3N2) virus. Frozen (−70°C) throat swab specimens were kindly provided by R. B. Couch and H. Six, Baylor College of Medicine, Houston, Tex. These specimens were from influenza surveillance studies and had previously been inoculated into tissue cultures for virus isolation. The treatment of throat swab specimens reinoculated into primary monkey kidney (PMK) tissue culture cells was described previously (15). Tissue culture fluids collected from PMK cells at 24 and 48 h after inoculation were diluted 1:2.5 in phosphate buffered saline and sonicated by using a probe sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). They were then further diluted to 1:5 with specimen diluent. Influenza viruses from the reference collection at the Centers for Disease Control, Atlanta, Ga., were used for hemagglutination inhibition testing and as controls in TR FIA and consisted of A/Bangkok/1/79 and A/Philippines/2/82 for hemagglutination inhibition and A/Ann Arbor/6/60 (H2N2)and B/Hong Kong/8/73 for TR FIA. Reference viruses were grown in the

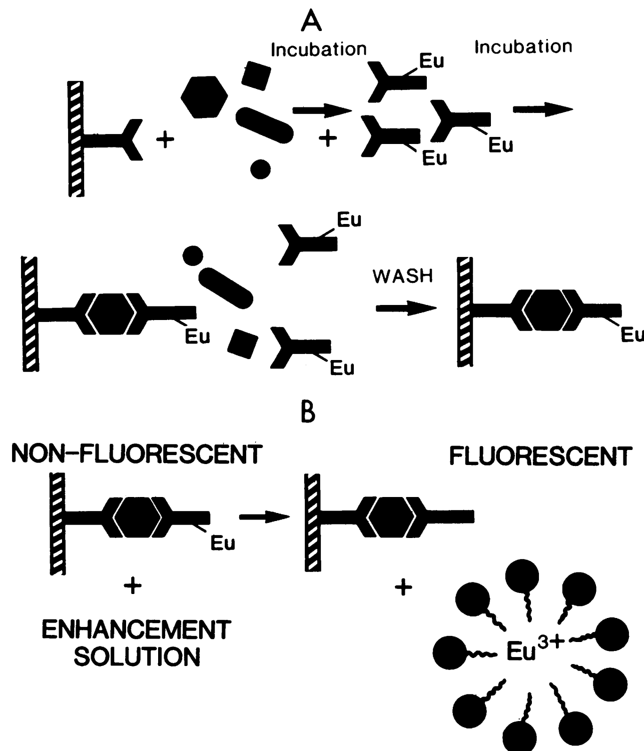


FIG. 1. Schematic illustration of the TR FIA procedure. (A) One-wash capture TR FIA; (B) fluorescence enhancement. [symbol], Surface of microtiter well; [symbol], monoclonal capture antibody; [symbol], influenza antigen; [symbols], other specimen components; [symbol], europium (Eu)-labeled monoclonal detector antibody; [symbol], enhancement solution.

allantoic cavities of 10-day-old chicken eggs. For quantitation of sensitivity of the influenza A assay, an influenza A nucleoprotein expressed by recombinant DNA in *Escherichia coli* and purified by affinity chromatography was kindly provided by G. G. Brownlee and I. Jones, University of Oxford, Oxford, England. The protein concentration of the nucleoprotein was determined in their laboratory.

**Monoclonal antibody purification and europium conjugation.** The monoclonal antibodies used in these studies have been previously described (15). The monoclonal antibody specificities were influenza A nucleoprotein (A1 and A3), influenza A matrix protein (A2), influenza B hemagglutinin (B1 and B4), and influenza B nucleoprotein (B2). The IgG fraction from ascitic fluids for each of the monoclones was purified by using a DEAE-Sephacel column equilibrated with 0.05 M Tris hydrochloride (pH 8.0). Protein fractions were eluted by using a continuous salt gradient (0.0 to 0.225 M NaCl) in 0.05 M Tris hydrochloride (pH 8.0). Peak protein fractions were pooled, concentrated, and precipitated overnight at 4°C with 40% saturated ammonium sulfate. The precipitated protein was resuspended in phosphate-buffered saline and dialyzed exhaustively against phosphate-buffered saline. The protein concentrations were determined by measuring $A_{278}$.

Each purified monoclonal antibody was conjugated with isothiocyanatophenyl-EDTA-europium by a previously described procedure (7). The europium chelate was provided by LKB-Wallac, Turku, Finland. Briefly, europium chelate was diluted in distilled water and adjusted by using a europium standard so that the europium/protein molar ratio was about 50:1. The protein and europium chelate were combined and carefully adjusted with 1 M $Na_2CO_3$ to pH 9.2, and the reaction mixture was held at 4°C overnight. Excess europium and protein aggregates were removed by gel filtration on a column (10 mm by 40 cm) consisting of 2 parts of Sephacryl S400 overlaid with 1 part of Sephadex G50 equilibrated with 0.05 M Tris (pH 7.75) buffer containing 0.154 M NaCl (Tris diluent). Each IgG protein molecule incorporated between 7.5 and 9.5 europium ions. The final protein concentration of each conjugate ranged from 160 to 300 μg/ml.

**TR FIA procedure.** The TR FIA as described is a two-incubation, one-wash procedure starting from the time of specimen addition to antibody-coated strips (Fig. 1A). Unless otherwise noted, this procedure was routinely used for testing both the NPS and tissue culture specimens. Capture antibodies were diluted in 0.5 M carbonate buffer (pH 9.6). Detector antibodies were diluted in either TR FIA Tris diluent A (Tris diluent containing 0.5% gelatin, 0.01% Tween 40, 20 μM diethylenetriamine pentaacetic acid, and 2% normal rabbit serum) or TR FIA Tris diluent B (as diluent A but with 2% bovine serum albumin substituted for 2% normal rabbit serum). (It has subsequently been found that satisfactory results can be obtained in TR FIA with the diluents described when gelatin is substituted for either normal rabbit serum or bovine serum albumin.) After optimization, monoclonal antibodies A3 and B4 were selected for capturing influenza A and B antigens, respectively, and were diluted to 4.0 and 2.0 μg/ml, respectively. The diluted

TABLE 1. Examples of TR FIA data obtained in the detection of influenza A viral antigens in NPS and in tissue culture inoculated with throat-swab specimens

| Identification of samples | No. of replicates | Mean cps ± SD | Ratio[a] |
|---|---|---|---|
| NPS | | | |
| Controls: | | | |
| Blank[b] | 12 | 353 ± 32 | |
| Diluent[c] | 6 | 441 ± 75 | 0.9 |
| Negative NPS pool[d] | 6 | 472 ± 73 | 1.0 |
| Sample test specimens[e]: | | | |
| Negative NPS | 2 | 498 ± 9 | 1.1 |
| High-positive NPS | 2 | 212,298 ± 13,375 | 450 |
| Low-positive NPS | 2 | 1,774 ± 147 | 3.8 |
| 48-h tissue culture specimens | | | |
| Controls: | | | |
| Blank[b] | 12 | 438 ± 29 | |
| Diluent[c] | 5 | 396 ± 49 | 1.0 |
| Sample test specimens: | | | |
| Negative[f] | 14[g] | 462 ± 72 | 1.2 |
| Influenza A | 2 | 145,014 ± 16,387 | 366 |
| Influenza B[h] | 14[g] | 416 ± 34 | 1.1 |

[a] cps with specimen per cps with diluent control or negative specimen control.
[b] Enhancement solution was added to empty wells.
[c] Specimen diluent was substituted for specimen.
[d] A group of NPS with no evidence of reactivity in either EIA or TR FIA with influenza A or B antibodies was pooled for use as a negative control.
[e] Specimens were considered positive if the cps for the specimen exceed the mean cps plus 4 SD for the diluent control or negative specimen pool.
[f] Tissue culture was inoculated with throat swab specimens from which no influenza viruses were isolated.
[g] Duplicate samples of seven different specimens were tested.
[h] Tissue culture was inoculated with throat swab specimens from which influenza B was previously isolated. The specimens were reactive in TR FIA for detecting influenza B viral antigens.

antibodies (0.25 ml per well) were added to the wells of microtiter strips (Flow Laboratories, Inc., McLean, Va.) and incubated overnight at room temperature in a moist atmosphere. The strips were washed three times with TR FIA wash (0.15 M NaCl, 0.05% Tween 20) by using a Nunc-Immuno Wash 12 (Nunc, Roskilde, Denmark) and were then postcoated by incubation overnight at room temperature with Tris diluent containing 0.1% gelatin. Strips were used within 1 week of preparation. Immediately before use, the antibody-saturated strips were washed twice with TR FIA wash. Diluted specimens (0.1 ml per well) were added to the strips containing influenza A or B capture antibody and incubated at room temperature for 30 min. Europium-conjugated detector antibodies A1 and B4 were diluted to 500 and 125 ng/ml, respectively, and 0.1-ml volumes were added to the influenza A or B strips, respectively, without washing off excess, unbound specimen. After 1 h of incubation at 37°C, the strips were washed six times, and 0.2 ml of enhancement solution (LKB Wallac) (7) was added. Strips were shaken for 10 min at room temperature to increase the dissociation of the europium from antibody chelate bound to captured antigen and to generate a new fluorescing europium chelate (Fig. 1B) which was measured with a model 1230 Arcus fluorometer (LKB Wallac). Examples of results for the assay are described in Table 1. The cutoff point for a specimen was recorded as positive in the TR FIA when the counts per second (cps) exceeded the appropriate control by more than 4 standard deviations (SD) of the mean control cps, after both cps had been adjusted by subtraction of the cps obtained with an enhancement solution blank. Diluent substituted for specimen was used as a control when testing tissue culture specimens, and both diluent and negative NPS pools were included when unknown NPS specimens were tested. For intertest comparisons, results were expressed as the ratio of cps observed with specimens versus their controls. The ratios were calculated after the cps were adjusted in the same way, except when the difference between the control and the enhancement solution blank was less than 100 cps. In this case, to minimize artifactual elevation of the ratios, gross counts were used to calculate ratios.

To optimize the conditions for the TR FIA, each of the monoclonal antibodies (A1, A2, or A3 for influenza A, or B1, B2, or B4 for influenza B) were evaluated as capture and as detector antibodies. Strips containing each antibody were used to capture homotypic viral antigen in NPS pools prepared from specimens previously found by EIA (15) to contain influenza A or B viral antigens, and each of the same antibodies, after conjugation with europium, was then used to detect the captured viral antigens. Thus, a total of nine antibody combinations were tested for both influenza A and B viruses. For detecting influenza A, the highest positive-to-negative ratios and lowest control counts were obtained with antibody A3 as the capture antibody and antibody A1 as the detector antibody; for influenza B, optimal results were obtained when antibody B4 was used for both antigen capture and detection.

The optimal coating of capture antibodies A3 and B4 was obtained when concentrations of 1,000 and 500 ng per well, respectively, were used. The actual concentrations of immunoglobulin adsorbed were not measured, however. With a constant optimal concentration of capture antibodies, the detector antibodies A1 and B4 gave optimal results at concentrations of 50 and 12.5 ng per well, respectively.

**EIA and RIA procedures for antigen detection.** In EIA culture confirmation tests, an indirect EIA procedure was used in which antigens were directly adsorbed onto plates and were detected with a pool of monoclonal antibodies (15). For detection of influenza A or B antigen in NPS, a slight modification of polyclonal capture EIA or RIA was used (11).

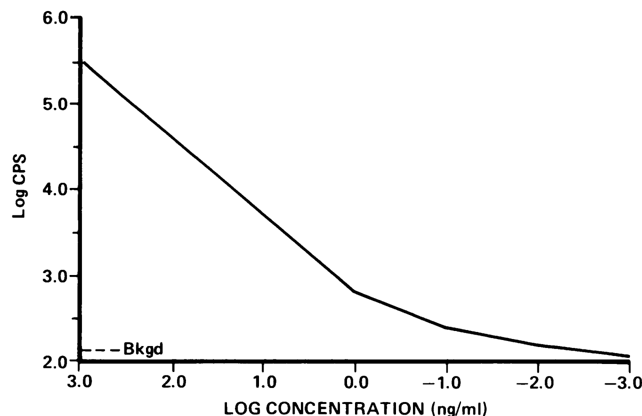


FIG. 2. TR FIA detection of purified influenza A nucleoprotein expressed by recombinant DNA in *E. coli*. The background value is the mean cps of six blank controls (background is 131 cps; mean + 4 SD = 238 cps).

## RESULTS

**Evaluation of the detection limits of the one-wash TR FIA for influenza A.** To determine the sensitivity of detection of the TR FIA for influenza A, we tested various dilutions of affinity-purified influenza A nucleoprotein (Fig. 2) that had been expressed by cloned recombinant DNA inserted into *E. coli*. Even at 100 pg/ml (10 pg per well), the cps were in excess of the cutoff value, and the ratio of the cps for the influenza A wells compared with that for the diluent controls was 1.8.

**Rapid detection of influenza viral antigens in NPS.** A group of 16 NPS previously shown to contain influenza A or B antigens by a polyclonal antibody capture EIA (11), or to be negative in that assay was tested for antigens reactive with influenza A or B reagents in TR FIA. Four of four influenza A specimens were positive in TR FIA with influenza A reagents, and five of five influenza B specimens were positive in TR FIA with influenza B reagents. Six of seven negative specimens were negative with both influenza A and B reagents. The discrepant specimen was negative by EIA but reacted with influenza A antibodies in TR FIA. All of the influenza A and B specimens were negative when tested in TR FIA with the heterotypic antibodies. These specimens were previously treated with 2% Tween 20; therefore, the presence of influenza viruses could not be confirmed by their isolation in tissue culture.

To determine the clinical sensitivity and specificity of the TR FIA, we applied it to a group of NPS from 96 patients for whom acute- and convalescent-phase serum specimens were also available, and who had been ill during one of two outbreaks of type A (H3N2) influenza. Because a comparison of hemagglutination inhibition and EIA tests for measurement of antibody rises showed few discrepancies and slightly higher sensitivity for the EIA, only results of the latter serodiagnostic tests were used for analysis. NPS from 33 patients were positive for influenza A by TR FIA. Of these, 32 were previously diagnosed as having influenza A

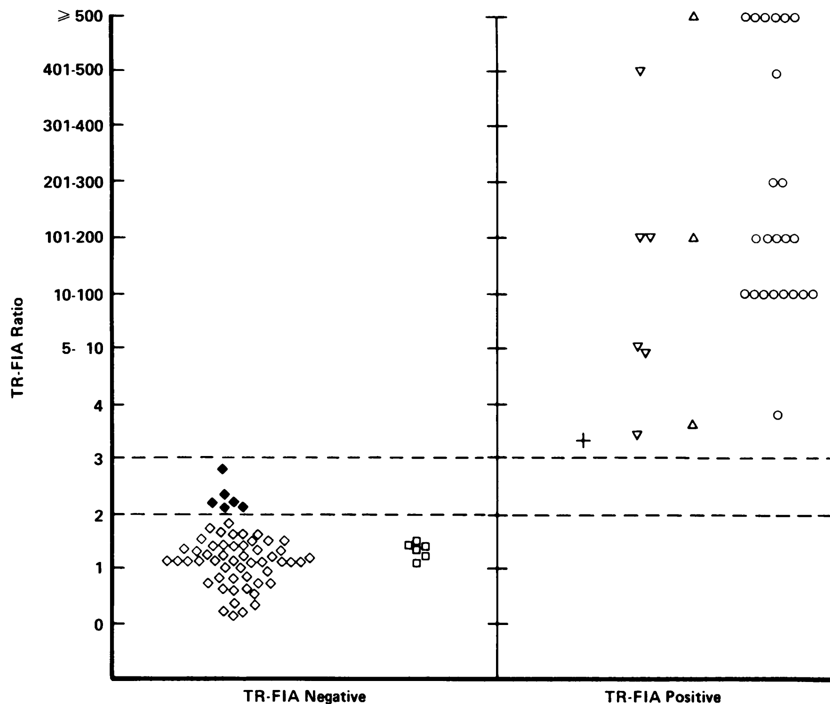


FIG. 3. Detection by TR FIA of influenza A antigens in NPS of patients sampled during influenza A (H3N2) outbreaks compared with other diagnostic tests. ◇, Influenza infection not confirmed by serodiagnosis or by any antigen detection immunoassay with NPS; ◆, as for ◇ but TR FIA ratios were >2 and TR FIA cps were less than 4 SD greater than control values; □, influenza infection detected by serodiagnosis, but not by TR FIA or other immunoassays; +, influenza infection detected at low level by TR FIA, but not by any other assay; ▽, influenza infection detected by serodiagnosis and TR FIA, but not by other immunoassays with NPS; △, influenza infection detected by TR FIA and other immunoassays with NPS, but not by serodiagnosis; ○, influenza infection detected by all assays.

infection by polyclonal antibody capture EIA or RIA tests for influenza antigen in NPS, or by having paired sera which showed a fourfold or greater rise in IgG or IgM antibody to influenza A as measured by EIA (Fig. 3). Of the 32 patients with serologic evidence of influenza A infection, 6 (20%) had antigen detected in their NPS by TR FIA but not by EIA or RIA. The single TR FIA-positive specimen that was not confirmed by other diagnostic methods produced relatively low cps with TR FIA (3.3 times the control), which either could be nonspecific or could indicate a greater sensitivity of the TR FIA procedure. By using the cutoff value of the mean cps for the diluent controls plus 4 SD, we found that NPS from 63 patients were negative by TR FIA. This included six specimens which produced cps in the TR FIA which were two- to threefold higher than the control. None of these NPS were positive by other antigen detection assays. However, six serum pairs from other patients in this group of 63 showed fourfold or greater rises in IgG or IgM antibody to influenza A as measured by EIA. In three of these six cases, the NPS were collected later than 48 h after the onset of illness. Making no allowance for the time of specimen collection, we found that TR FIA had a specificity of 95% and sensitivity of 85% for confirming influenza A infection when comparing TR FIA diagnostic rates with the rates obtained by measuring antibody rises in paired sera.

The NPS for each of these 96 patients were also tested by TR FIA for evidence of influenza B antigens. An NPS from one patient was positive for influenza B by TR FIA, although the other assays failed to provide evidence for influenza B infection.

**Comparison of endpoint titers obtained with TR FIA and polyclonal capture EIA.** The sensitivity of the TR FIA was compared with that of a polyclonal capture EIA (11) by preparing pools of either influenza A- or influenza B-positive NPS and titrating each with the two procedures. Both the influenza A and B NPS pools were reactive in TR FIA in their type-specific antibodies when they were diluted to 1:10,000. In contrast, the same specimens were reactive in EIA at only a 1:100 dilution.

**Rapid culture confirmation of influenza A and B viruses in throat swab specimens.** Supernatants from PMK cells inoculated with throat swab specimens and collected at 24 and 48 h after inoculation had been stored for about 1 year after previous EIA tests to detect influenza virus antigen (15). These stored tissue culture specimens were thawed and examined for their reactivity in a TR FIA procedure. In contrast to the usual TR FIA procedure, the microtiter wells were washed after incubation with the culture supernatants and before the addition of the europium-labeled antibodies so that the assay was analogous to standard EIA procedures. At 24 h after infection of tissue culture cells with swabs from patients actually infected with influenza A (H1N1 or H3N2), 45 and 29% of the harvests, respectively, were positive when tested by TR FIA (Fig. 4A and B), and at 48 h, more than 80% of the H1N1 and H3N2 isolates were positive by TR FIA. For influenza B isolates, the corresponding results were 10 and 80% positive by TR FIA at 24 and 48 h postinoculation (Fig. 4C). The results for influenza A indicated slightly higher sensitivity of antigen detection early after inoculation of cell cultures than had been previously observed with the same tissue culture harvest previously tested by an EIA in which the antigens had been adsorbed to

the plastic plates before detection with monoclonal antibodies (Fig. 4A and B) (15). This was not the case with the results with influenza B (Fig. 4C).

## DISCUSSION

TR FIA takes advantage of the large Stokes' shift and long decay time of europium chelates when used as fluorescent probes (7). These factors enabled the development of a fluorometer which has high statistical precision with a 1-s counting time (14). Europium chelate conjugates are stable and nonisotopic reagents; thus, assays that use these reagents are easily standardized and have no known biological risks.

Previous comparison of TR FIA procedures with RIA procedures for detecting influenza viruses in NPS showed sensitivities that were nearly identical (3). However, in both procedures a complex immunoassay was used, and the authors suggested that greater sensitivities with the TR FIA might be achieved in direct, capture assays by using monoclonal antibodies.

Monoclonal antibodies to type-specific antigens present in all influenza A and B viruses have recently been shown to be useful for detecting viruses in both original specimens and in tissue culture isolates by EIA or indirect fluorescent antibody tests (9, 12, 13, 15). We, therefore, investigated whether these antibodies would be useful reagents in the TR FIA procedure.

When TR FIA was compared with a highly sensitive serodiagnostic assay, the TR FIA gave positive results for influenza A antigen in NPS of 85% of patients who had serologic evidence of influenza A infection, with a 95% specificity. Compared with a previously used EIA procedure, TR FIA detected 20% more positive NPS specimens among patients wih serologic evidence of infection. It is possible that higher sensitivity of the TR FIA would be found if only NPS specimens that were collected from patients within 48 h of onset of illness were tested. Because serologic diagnosis of influenza infection is generally more sensitive than virus isolation for confirmation of infection, the overall results show a very high efficiency of antigen detection in NPS by the TR FIA assay, without worrisome levels of nonspecificity.

Although direct detection of influenza antigens in clinical specimens is a desirable procedure when specific actions depend on the result (e.g., therapy with an antiviral drug, or withholding of antibiotics in the absence of evidence for secondary bacterial complications), in many cases a delay of 1 or 2 days in obtaining laboratory confirmation does not preclude appropriate clinical action. For example, when influenza A viruses are known to be in the community, and increased numbers of cases of influenzalike illness occur among residents of a high-risk institution, it has been proposed that amantadine, the currently available antiviral agent, be provided to exposed residents for 3 days, pending laboratory confirmation (2). This practical approach to controlling influenza A infections in high-risk institutions allows time for rapid culture confirmation, but even culture confirmation depends on a sensitive assay for antigen detection.

Furthermore, while the success of direct antigen detection procedures with NPS is very dependent on obtaining and efficiently processing high-quality clinical specimens, nose or throat swabs for culture confirmation can be readily collected, transported (if necessary) by overnight mail, and handled in large numbers without special processing, such as ultrasonication. In addition, isolation of the virus in cultures

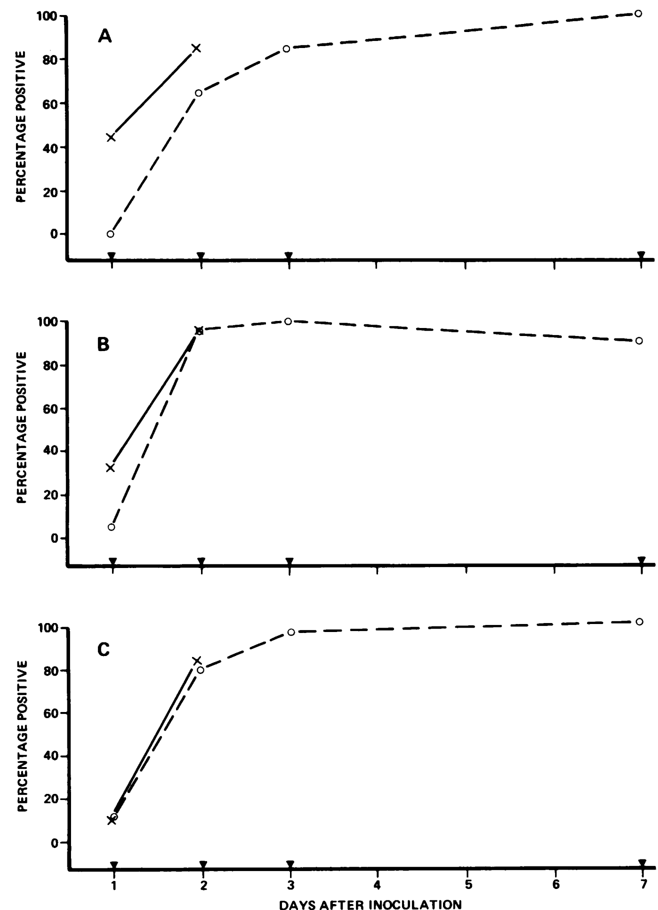


FIG. 4. Detection of influenza A or B antigen production at different times after inoculation of tissue culture with throat swabs. Results are expressed as the percentage of isolates detected compared with the maximum at 7 days after inoculation. (A) Influenza A (H1N1) virus; (B) influenza A (H3N2) virus; (C) influenza B virus. Symbols: ×——×, TR FIA; ○— — —○, EIA.

provides the materials necessary for further characterization of antigenic or biologic properties. Such reference analysis is important for both quality control and epidemiologic purposes, particularly when influenza epidemics first begin.

In this study, more than 80% of the inoculated tissue cultures that ultimately grew influenza A (H1N1), influenza A (H3N2), or influenza B virus was positive by TR FIA within 48 h of specimen inoculation despite the fact that suboptimal, stored specimens were used. Previously, 48 or 72 h of incubation was required to obtain this level of detection when the same specimens were tested by EIA (15). Under ideal culture conditions, both the TR FIA and EIA results would probably be higher, and further field testing is desirable. For diagnosing outbreaks in which multiple specimens can be collected from acutely ill persons, the sensitivity of the TR FIA for antigen detection appears to provide practical, useful results even within 24 h after specimen inoculation.

Alternate EIA test configurations that might increase the sensitivity of antigen detection by EIA methodology are feasible. The present results, however, strongly suggest that TR FIA, with broadly reactive influenza monoclonal antibodies, provides the most rapid, sensitive, and practical procedure yet described for objective, quantitative identifi-

cation of influenza A or B viral antigens for either culture confirmation or direct detection in original nasopharyngeal aspirates. For laboratories with the potential for high throughput of specimens, TR FIA would appear to offer worthwhile advantages over fluorescence microscopy, particularly when used in conjunction with TR FIA procedures for other respiratory illnesses such as respiratory syncytial virus and parainfluenza virus, which have been developed (4) or are planned for the near future. Introduction of this new antigen-detection technology for either rapid diagnosis with clinical specimens or rapid culture confirmation should, in the long run, offer worthwhile savings in cost compared with traditional virus culture detection methods. This will allow the microbiology laboratory to efficiently handle large numbers of specimens for patient diagnosis and surveillance programs, thus facilitating the introduction of active influenza control programs at the community level.

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