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# Rapid Diagnosis of Influenza A Infection by Direct Immunofluorescence of Nasopharyngeal Aspirates in Adults

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The efficacy for direct immunofluorescence of a commercial conjugate for influenza A virus prepared against whole A/Udorn ($H_3N_2$) virus was studied. The conjugate was specific for influenza A virus, but its sensitivity varied depending upon the strain of influenza A tested. Nasopharyngeal aspirates collected from 25 patients during an outbreak of influenza were examined for viral antigen with the conjugate and inoculated onto monkey kidney (MK) cells for virus isolation. Fifteen patients had isolates for influenza A/USSR/90/77 ($H_1N_1$); nasopharyngeal secretions were fluorescent antibody positive in 12. Fluorescent antibody was copositive with culture in 11/15 patients (73.3%) and conegative in 9/10 (90%). The one fluorescent antibody-positive, culture-negative patient had negative serology for influenza A and the fluorescent antibody result was considered to be a false positive. At a 1:10 dilution, the conjugate stained nasopharyngeal and MK cells infected with A/USSR ($H_1N_1$) 2 to 3+, whereas cells infected with $H_3N_2$ virus stained 4+. A conjugate made specifically against the ribonucleoprotein antigen, which is universal to all influenza A strains, may improve the sensitivity of the direct immunofluorescent test.

The detection of antigen by fluorescein-labeled antibody (FA) was described by Coons et al. in 1941 (2). Liu (13) first applied this technique for rapid viral diagnosis on clinical specimens. However, for several years FA did not enjoy wide use in viral diagnosis. Recently, there has been a surge of interest in rapid viral diagnosis, and both a European and a Pan-American group for rapid viral diagnosis have been formed. This has resulted in renewed interest in immunofluorescence as a technique with wide potential.

The advantage of rapid diagnosis is apparent in several areas. Antiviral agents are available for some diseases—amantadine for influenza A (4, 11, 15), adenine arabinoside for herpes simplex encephalitis (19), and interferon for disseminated varicella zoster (14). In the immunocompromised host, early diagnosis of a viral infection can guide management away from unnecessary antibiotics and open-lung biopsy and towards specific antiviral chemotherapy where available. In the hospital, rapid diagnosis can help control nosocomial spread of viral disease (7, 9). Finally, early diagnosis is important in alerting public health officials to the possible onset of an epidemic and the institution of proper control measures.

Several investigators have studied immunofluorescence for rapid diagnosis of viral infections (3, 5, 8, 12, 16). Generally, an indirect immunofluorescence test has been used and the reagents have been prepared by the investigators rather than by a commercial source. In this study, we examined nasopharyngeal (N-P) secretions for influenza A virus with a commercially available direct conjugate and compared the result with tissue culture isolation.

## METHODS

**Fluorescent conjugate.** The influenza A direct fluorescein conjugate was supplied by Flow Laboratories, Rockville, Md. It was prepared in rabbits with whole A/Udorn /307/72($H_3N_2$) virus. Pooled preimmunization serum from the same rabbits was also fluorescein labeled to serve as a control. The conjugated immune serum (conjugate) and preimmunization serum control (control serum) were absorbed with whole human embryo (Flow 5000), baby hamster kidney, Vero, Hep 2, and WI 38 cell cultures.

**(i) Laboratory tests for specificity and sensitivity.** The conjugate was tested against influenza A/Victoria/3/75($H_3N_2$) and A/USSR/90/77 ($H_1N_1$) grown in primary rhesus monkey kidney (MK) tissue culture. As controls: (i) the preimmunization control serum was tested against A/Victoria and A/USSR; (ii) the conjugate and control serum were tested against uninfected MK cells and MK cells infected with influenza B/Hong Kong/5/72; parainfluenza types 1, 2, and 3; respiratory syncytial; and mumps viruses; (iii) a

single-step blocking test was done with serum with a high complement-fixing antibody titer to influenza A virus (6).

As a test of the sensitivity of the conjugate for detecting various influenza A strains, it was tested against A/Sw/15/31 ($Hsw_1N_1$), A/PR/8/34($H_0N_1$), $A_1/FM_1/47$ ($H_1N_1$), and A/NJ/8/76($Hsw_1N_1$) grown in MK tissue culture.

**(ii) Clinical application. Patient selection.** During February 1978, an outbreak of influenza A infection occurred in Philadelphia. This gave us an opportunity to study the conjugate for rapid diagnosis on clinical specimens.

All patients were seen over a 2-week period (13 to 24 February 1978) toward the end of the approximately 1-month-long epidemic. They came from the Walk-In (outpatient) and Employee Health Clinics of the Hospital of the University of Pennsylvania, and the Nursing Students' and Students' Health Clinics of the University of Pennsylvania. Patients were seen by one of us (J. D.) and were selected on the basis of symptoms and signs compatible with a diagnosis of influenza, provided that the patient was seen within 96 h of clinical onset. Twenty-five patients met these criteria.

**Collection and processing of clinical specimens.** N-P secretions were collected and processed by using minor modifications of the method described by Gardner and McQuillin (5). Briefly, N-P aspiration of each nostril was performed with a portable aspirator and a no. 10 French polyethylene suction catheter with a secretion trap attached. The secretions were washed through the catheter with Hanks balanced salt solution (pH 7.0) supplemented with 0.5% gelatin and 10% sucrose phosphate glutamine. The specimen was placed on ice and promptly processed on return to the laboratory—usually within 3 to 4 h of collection.

The N-P aspirates were centrifuged at 600 × *g* at 4°C in a Sorvall R-3 centrifuge, and the supernatant fluid was removed for culturing. The pellets, consisting of mucus and cells, were washed several times with 2 to 3 ml of phosphate-buffered saline to remove the mucus. This step required painstaking care, and each specimen was handled individually. Extended washings were necessary with some specimens. The cells devoid of mucus were finally suspended in a few drops of phosphate-buffered saline (0.3 to 0.5 ml) and dropped by Pasteur pipettes into wells of Teflon-coated, acetone-resistant 10-well microscope slides (Cel-line Associates, Minotola, N.J.). At least one slide for FA staining and one for quantitation of cells by May-Grünwald-Giemsa staining were prepared from each specimen.

**FA testing of specimens.** Slides were air dried and fixed in acetone at 4°C. The conjugate was added at a 1:10 dilution, and the slides were incubated at 37°C for 30 min. They were washed three times, 5 min each, with phosphate-buffered saline with gentle agitation, rinsed for 2 min in deionized distilled water, air dried, and mounted with Elvanol with a no. 2 cover slip (24 by 50 mm; A. H. Thomas Co., Philadelphia, Pa.). The same investigator (H. F.) read all the slides by fluorescent microscopy, unaware of which wells were stained with conjugated test or control serum. They were examined under a Leitz Ortholux II fluorescent microscope with an HBO 200 W/4 Hg lamp.

**Culture of N-P secretions.** Supernatant fluid from N-P aspirates was incubated with gentamicin (50 μg/ml), penicillin (500 U/ml), and amphotericin B (10 μg/ml) for 20 min at room temperature. A 0.2-ml amount of each aspirate was then inoculated into duplicate MK culture tubes maintained on Eagle minimum essential medium containing 2% fetal calf serum, 1% glutamine, and gentamicin, penicillin, and amphotericin B at final concentrations of 10 μg, 150 U, and 1.5 μg/ml, respectively. Uninoculated MK tubes served as controls. The cultures were incubated at 33°C in a roller drum.

Cultures were tested for the presence of virus by hemagglutination and hemadsorption by standard techniques (17) at 10 to 14 days after inoculation, or earlier if a cytopathic effect was evident. When negative, cells and the supernatant fluids were subpassed once.

**Viral identification.** Each isolate was identified by the hemagglutination inhibition test with World Health Organization reference antisera and their control antigens for influenza A/USSR/90/77($H_1N_1$), A/Victoria/3/75($H_3N_2$), and B/Hong Kong/5/72.

**Serology.** Paired sera were available from 22 patients taken on admission and 2 to 7 weeks later. They were tested for antibodies to an isolate obtained from one patient after the virus had been subpassed in embryonated chicken eggs. Hemadsorption-neutralization tests were performed on these sera in Madin Darby canine kidney (MDCK) cell cultures by techniques previously described (18).

## RESULTS

**Fluorescent conjugate.** The fluorescent conjugate was tested against A/Victoria/3/75 ($H_3N_2$) grown in MK cell cultures. The intensity of the fluorescence (on a scale of 0 to 4) was 4+ at 1:10, 2 to 3+ at 1:20, 2+ at 1:40, 1+ at 1:80, and negative at higher dilutions. In general, there was a correlation between the number of tissue culture cells with positive fluorescence and the intensity of staining. A 1:10 dilution was considered optimal, and this dilution was used in subsequent tests for specificity.

As shown in Table 1, the conjugate was specific for influenza A virus. It reacted with MK cells infected with several strains of influenza A virus but did not react with uninfected MK cells or MK cells infected with influenza type B; parainfluenza types 1, 2 or 3; mumps; or respiratory syncytial viruses. The conjugated serum control did not react with uninfected MK cells or MK cells infected with any of the viruses tested. In addition, serum with a high complement fixation antibody titer to influenza A virus successfully blocked the specific fluorescence of the conjugate with $H_3N_2$-infected MK cells. A second serum without antibody to influenza A had no blocking effect.

Table 1 also demonstrates that there was

some variation in the sensitivity of the conjugate for the several strains tested. The $H_3N_2$ strains and the most recent swine strain (A/NJ/8/76) reacted best at ≥3+ intensity of fluorescence with the conjugate at a 1:10 dilution. $H_1N_1$ strains were intermediate (2 to 3+) and an $H_0N_1$ strain stained poorly (1 to 2+).

**FA testing of clinical specimens.** Of the 25 N-P aspirates tested by direct FA, 12 were positive with an intensity of 2 to 3+. The appearance was stippled with mainly cytoplasmic fluorescence. The optimal dilution of the conjugate for staining clinical specimens was 1:10. In general, two or more, though seldom many, cells were positive on each smear. All positives, however, were detected on reading of the first slide and staining additional slides did not increase the number of positive results. The conjugated serum control was negative against all clinical specimens.

In five cases, slides stained for FA had very few cells. In three of these cases, the matching slides stained with May-Grünwald-Giemsa also had few cells, indicating a poor original sample. In two cases, many cells were seen on Giemsa staining. Presumably, in these latter cases, the cells were lost during preparation for FA. Elapsed time from the beginning of the preparation of the clinical specimen to the reading of the results was 4 to 5 h. For some specimens, results were available the same day the sample was collected.

TABLE 1. *In vitro testing of the fluorescent conjugate*

| Agent in monkey kidney cells | Conjugated antiserum (1:10) | Preimmune conjugated serum (1:10) |
|---|---|---|
| A/Victoria/3/75 ($H_3N_2$) | 4+ | Negative |
| A/USSR/90/77 ($H_1N_1$) | 2–3+ | Negative |
| $A_2$/HK/8/68 ($H_3N_2$) | 3+ | Negative |
| A/SW/15/31 ($Hsw_1N_1$) | 2–3+ | Negative |
| A/NJ/8/76 ($Hsw_1N_1$) | 3+ | Negative |
| A/FM/1/47 ($H_1N_1$) | 2–3+ | Negative |
| A/USSR/77 ($H_1N_1$) | 2–3+ | Negative |
| A/PR/8/34 ($H_0N_1$) | 1–2+ | Negative |
| Uninfected cells | Negative | Negative |
| B/Hong Kong/5/72 | Negative | Negative |
| Parainfluenzae 1, 2, 3 | Negative | Negative |
| Mumps | Negative | Negative |
| Respiratory syncytial virus | Negative | Negative |

TABLE 2. *Correlation between immunofluorescence (FA) and viral isolation*[a]

| Immunofluorescence | Culture Positive | Culture Negative |
|---|---|---|
| FA positive | 11 | 1 |
| FA negative | 4 | 9 |

[a] Copositivity (11/15) = 73.3% (after Gardner and McQuillin [5]); conegativity (9/10) = 90%. Overall correspondence (20/25) = 80%.

**Virus isolation and identification.** Of the 25 N-P aspirates inoculated onto MK cells, 15 were positive. All positives were identified as A/USSR/90/77 ($H_1N_1$) by the hemagglutination inhibition tests for each one reacted to the same titer with the A/USSR/90/77 ($H_1N_1$) antiserum as did the control homologous reference antigen. None reacted with either of the other two test antisera.

**Culture versus FA.** Table 2 shows the correlation between FA and tissue culture techniques for influenza diagnosis. Of the 12 specimens positive by FA, 11 also yielded virus by tissue culture. Four culture positive specimens, however, were missed by FA, making its sensitivity 73.3% (11/15). The one who was FA positive but culture negative was a 28-year-old patient with hemadsorption-neutralization antibodies in both serum specimens. This case is regarded as an FA false positive. Overall, FA results corresponded with those of tissue culture in 20/25 (80%) of cases.

**Serology.** Paired sera were available from 13 of the 15 patients who yielded virus. All but two of them showed seroconversion, the convalescent serum titers ranging from 1:8 to 1:64 with a geometric mean titer of 1:16. Of the 10 patients from whom virus was not isolated, 2 nevertheless showed antibody rises, 4 had antibodies on their first and second serum specimens, 2 were completely negative, and no serum pairs were available from the remaining 2 persons. Thus, of the 25 patients 17 sustained infection with the USSR variant as proven by virus isolation and/or seroconversion, and four had evidence of past infection with the same virus. It should be noted that two of these four were born in 1949 or 1950, and their antibodies could have reflected experience with a similar virus during their childhood.

**Clinical data.** The oldest patient in the study was 28 years old: no culture-positive patient was older than 25 years. This was not a product of the population sampled, for although the Nursing Student and Student Health Clinics are biased in favor of persons <25 years old, the Employee Health and Walk-In Clinics, which together supplied 52% (13/25) of the study pa-

tients, have patients of all ages. The symptoms and signs were typical of influenza infection except that intestinal symptoms, especially nausea and vomiting, were frequent (occurring in 11 study patients, 5 of whom were culture positive). The illness was mild, in general. Only one patient was hospitalized in the acute phase for severity of symptoms (marked malaise and temperature >40°C), and no one was treated for complications.

Fourteen culture-positive patients were seen in follow-up, and all reported a return to normal health by 7 to 10 days after clinical onset.

## DISCUSSION

A conjugate which is used to test clinical material must be standardized against known positive and negative clinical specimens. In this study, we tested a commercially prepared influenza A conjugate for use in rapid diagnosis by staining nasopharyngeal secretions collected from patients some of whom were infected with influenza A virus. The conjugate was applied at a working dilution of 1:10. This was the optimal dilution for producing the most intense fluorescence with the least background staining on tissue culture cells infected with $H_1N_1$ virus, and on clinical specimens. Comparing results of FA on N-P secretions with viral isolation, there was copositivity in 73.3% (11/15) and conegativity in 90% (9/10). The one FA-positive but tissue culture-negative patient had negative serology and is considered an FA false positive.

Our data correspond with those of several other investigators who have applied immunofluorescence to nasopharyngeal secretions for the diagnosis of influenza A infection (3, 5, 8, 12, 13, 16). FA in this situation has proven to be highly specific when compared with serology or isolation: the sensitivity (copositivity), however, has varied from 63% (3) to 94% (5) when compared with standard techniques.

Criteria regarding acceptable sensitivity of a conjugate have been vague. The conjugate used in the present study reacted with tissue culture cells infected with A/Victoria, an $H_3N_2$ virus, with a 2 to 3+ intensity at 1:20, but with A/USSR ($H_1N_1$) it stained 2 to 3+ at a 1:10 dilution (both in tissue culture and N-P aspirates). This could be expected since the conjugate contained antibodies to both the surface antigen and the RNA protein components of the virus and, conceivably, titers to the former might have been higher. The staining observed with the non-$H_3N_2$ strains detected only the RNA protein. Although correlation of FA staining of N-P secretions with viral isolation was good, we did encounter four false negatives for a failure rate of 26.7% (4/15). A better result might have been achieved had the conjugate contained antibodies against the surface antigen of the influenza A virus causing infection. Sensitivity testing of the conjugate with MK cells infected with several strains of influenza A (Table 1) appear to support this view. Perhaps for rapid diagnosis of any influenza A infection, a high-titered conjugated antiserum prepared against the RNA protein of influenza A, which is the same for all type A viruses, would be the reagent of choice. The same can be said for a diagnostic test for type B influenza virus as well. Methods for producing high-titrated sera containing only antibodies to the RNA protein of influenza viruses are known (1, 10). For strain diagnosis by FA, such sera might be supplemented by the use of reliably sensitive reagents containing antibodies to surface antigens of strains circulating at the time.

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### LITERATURE CITED

1. **Center for Disease Control.** 1975. Advanced laboratory techniques for influenza diagnosis. Immunology series no. 6 procedural guide. U.S. Department of Health, Education and Welfare, Public Health Service. Center for Disease Control, Atlanta.
2. **Coons, A. H., J. H. Creech, and R. N. Jones.** 1941. Immunological properties of an antibody containing a fluorescent group. Proc. Soc. Exp. Biol. Med. **47**:200–202.
3. **Fedova, D., and L. Zelenkova.** 1965. The use of fluorescent antibody method for rapid identification of the $A_2$ influenza virus in nasal smears by the fluorescent antibody technique. J. Hyg. Epidemiol. Microbiol. Immunol. **9**:135–146.
4. **Galbraith, A. W., J. S. Oxford, G. L. Schild, C. W. Potter, and G. I. Watson.** 1971. Therapeutic effect of 1-adamantanamine hydrochloride in naturally occurring influenza $A_2$ Hong Kong infection. Lancet **ii**:113–115.
5. **Gardner, P. S., and J. McQuillin.** 1974. Rapid viral diagnosis. Application of immunofluorescence. Butterworth & Co., London.
6. **Goldman, M.** 1968. Fluorescent antibody methods, p. 169. Academic Press Inc., New York.
7. **Hall, C. B., and R. G. Douglas Jr.** 1975. Nosocomial influenza infections as a cause of intercurrent fevers in infants. Pediatrics **55**:673–677.
8. **Hers, J. F., L. P. Van der Kuip, and N. Masurel.** 1968. Rapid diagnosis of influenza. Experience in a winter outbreak. Lancet **i**:510–511.
9. **Hoffman, P. C., and E. Dixon.** 1977. Control of influenza in the hospital. Ann. Intern. Med. **87**:725–728.
10. **Lief, F. S.** 1963. Antigenic analysis of influenza viruses by complement fixation. VII. Further studies on production of pure anti-S serum and on specificity of the A S antigens. J. Immunol. **90**:172–177.
11. **Little, J.W., W. J. Hall, and R. G. Douglas, Jr.** 1976. Amantadine effect on peripheral airways abnormalities in influenza. Ann. Intern. Med. **85**:177–182.
12. **Liu, C.** 1961. Diagnosis of influenzal infection by means of fluorescent antibody staining. Am. Rev. Respir. Dis. **83**: S130–133.

13. **Liu, C.** 1956. Rapid diagnosis of human influenza infection from nasal smears by means of fluorescein-labelled antibody. Proc. Soc. Exp. Biol. Med. **92**:883–887.
14. **Merigan, T. C., K. H. Rand, R. B. Polland, P. S. Abdullah, G. W. Jordan, and R. P. Fried.** 1978. Interferon for the treatment of herpes zoster in patients with cancer. N. Engl. J. Med. **298**:981–987.
15. **Muldoon, R. L., E. D. Stanley, and G. G. Jackson.** 1976. Use and withdrawal of amantadine chemoprophylaxis during epidemic influenza A. Am. Rev. Respir. Dis. **113**:487–491.
16. **Olding-Stenkvist, E., and M. Grandien.** 1977. Rapid diagnosis of influenza A infection by immunofluorescence. Acta Pathol. Microbiol. Scand. Sect. B **85**:296–302.
17. **Robinson, R. Q., and W. R. Dowdle.** 1969. Influenza viruses, p. 414–433. *In* E. H. Lennette and N. J. Schmidt (ed.), Diagnostic procedures for viral and rickettsial infections, 4th ed. American Public Health Association, Inc., New York.
18. **Todd, J. D., F. S. Lief, and D. Cohen.** 1970. Experimental infection of ponies with the Hong Kong variant of human influenza virus. Am. J. Epidemiol. **92**:330–336.
19. **Whitley, R. J., S.-J. Soong, R. Dolin, G. J. Galasso, L. T. Ch'ien, C. A. Alford, and the Collaborative Study Group.** 1976. Adenine arabinoside therapy of biopsy-proved Herpes simplex encephalitis. N. Engl. J. Med. **297**:289–294.