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Rapid Diagnosis of Human Influenza Infection from Nasal Smears by Means of Fluorescein-Labeled Antibody.*† (22642)

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Laboratory diagnosis of influenzal infection is usually carried out by means of retrospective serological reactions or by isolation of the virus from throat washings. Attempts have been made to shorten the time for diagnosis. Hummeler *et al.*(1) used the complement fixation test with known serum and the soluble antigen from primary isolation of the virus in chick embryos. He reported that typing of the virus was successful in 48 to 72 hours after collection of specimens. Fazekas de St. Groth(2) found that the "inhibitory index" to influenza virus of human nasal mucus was significantly increased during the acute stage of infection, allowing an early presumptive diagnosis of influenzal infections.

In the study of influenzal infection in ferrets by means of fluorescein-labeled antibody, it was possible to make a rapid diagnosis of influenzal infection during the acute stage of illness by detecting specific viral antigen in the desquamated nasal epithelial cells and macrophages in the nasal smears(3). This paper reports the application of this method to human influenzal infection. The results indicate that, although the staining of nasal smears by fluorescein-labeled antibody is less

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sensitive than the standard hemagglutination-inhibition test for the diagnosis of influenzal infection, it is much quicker. A diagnosis can be made on the same day as the collection of the nasal washings.

Materials and methods. Most of the patients were young male students admitted to the infirmaries of Harvard University, Cambridge, Mass. or Phillips Exeter Academy in Exeter, N. H. with a clinical diagnosis of influenza or acute upper respiratory infection. The patients at Harvard were examined by the author and the patients in Exeter were examined by Dr. James T. Heyl. On all patients, nasal washings and a sample of venous blood were collected during the first visit while they were at the infirmary. The convalescent blood samples were collected 2 to 3 weeks after they were discharged.

Preparation of nasal smears. Nasal washings were performed with the patient in a supine position, and the neck fully extended. With a 10 ml pipette, 5 ml of normal 0.85% saline was introduced into each nostril. (In a few cases, beef heart infusion broth was used instead of saline.) After instillation of the solution, the patient was allowed to sit up to let the saline drip out and the liquid was collected into a Petri dish. Usually about 4 to 5 ml of liquid was recovered from both nostrils. About one ml of the washings was put into a test tube, sealed and stored in a dry CO_2 box to be used for virus isolation. The remainder was transferred to a test tube and centrifuged at about 1,000 rpm for 5 minutes. The supernatant fluid was removed by a capillary pipette, leaving about 0.2 ml of liquid. The cellular sediment was then dispersed in this small volume of saline. A drop of the cell suspension was spread over an area of about 1 cm in diameter on a 3 x 1 inch glass slide. Four to 5 slides were made from each nasal washing. The slides were dried in air at room temperature and then kept in the refrigerator at 4°C. Slides may also be dried directly in the refrigerator. In a few patients, a small cotton swab wetted with saline was introduced into the upper nasal passage with the aid of a nasal speculum and a head mirror. The nasal turbinates were touched by the swab, and smears were made by rolling the swab on a glass slide.

Fluorescein-labeled antisera were prepared according to the method of Coons and Kaplan(4). The specific influenza sera against influenza A (Farrington and Fm_1 strains) and the Lee strain of influenza B viruses were prepared in rabbits as described previously(3). Before use as a histochemical stain on nasal smears, the fluorescent antibody solutions were absorbed twice with ferret liver powder and once with rabbit bone marrow powder. The latter absorption is for the removal of non-specific fluorescence in white blood cells, especially the granulocytes(5).

Fixation, staining and examination of slides. Nasal smears were fixed in acetone for 10 minutes and dried in a 37°C oven for 20 minutes. Usually 2 slides were fixed and dried in this way. For staining, one slide was treated with a drop of influenza A fluorescein-labeled antibody solution for 30 minutes at room temperature; the other slide was treated in a similar manner with a fluorescent influenza B antibody. Afterwards, the slides were washed in buffered saline (pH 7.0) for 10 minutes and then mounted under a coverslip in buffered glycerol (pH 7.0). The slides were given only a code number while being examined under the fluorescence microscope and decoded afterwards. All slides were examined by one person and recorded as positive when several to many definite specifically yellow-green fluorescent cells were seen; doubtful when only 1 or 2 fluorescent cells were present; and negative when no fluorescent cells were found.

Isolation of virus. For the isolation of influenza virus from throat washings, the method of amniotic inoculation as described by Beveridge and Burnet(6) was followed. 1,000 units of penicillin and 500 μg of streptomycin were added to each ml of inoculum.

Hemagglutination - inhibition (HI) tests were performed by the technic described as the Standard Reference Test for Influenza Studies(7). All sera were inactivated at 56°C for 30 minutes, then treated with 4 volumes of crude cholera vibrio filtrate for 18 hours at 37°C to remove the non-specific in-

hibitors(8). For each test, 4 strains of viral antigens were used: PR8, Fm_1, Lee B, and a current strain of either influenza A or B viruses isolated during the epidemics. A 4-fold or greater increase of antibody titer in the convalescent serum was considered significant.

Analysis of laboratory data. The coded nasal smears stained with fluorescent antibody solutions were usually examined within 24 hours after specimens were collected. The results were then decoded and recorded. Two to 3 weeks later, when acute and convalescent sera were available and HI tests and virus isolation experiments completed, they were compared with the results from the examination of the smears. Influenzal infection was considered confirmed in those patients who had a 4-fold or greater rise of HI antibody titer during convalescence or a positive virus recovery from the nasal washings. Degree of positive correlation as expressed by the ratio of number of positive nasal smears to total number of confirmed cases of influenza is a measure of sensitivity of the test of examination of nasal smears by means of fluorescein-labeled antibody.

Results. It was not the aim of this study to carry out a detailed epidemiological survey of influenza but to attempt to develop a simple, rapid diagnostic method for this disease. Therefore, only a limited number of patients was selected, enabling us to undertake a complete laboratory diagnosis on each patient.

Influenza A-prime epidemics occurred in New England during 1952-3. Twenty patients admitted to the Harvard Stillman Infirmary between the last week of Jan. and the first 2 weeks of Feb. 1953, were studied. The results are summarized in Table I. For fluorescein-labeled antibody staining, only the conjugate against the Farrington strain of influenza virus was used, because this was the only conjugate available at that time. This strain of virus is closely related to the classical PR8 strain of influenza A virus, as reported previously(10). In the hemagglutination-inhibition tests, 4 strains of influenza viral antigens (PR8, Fm_1, 53-Ben and Lee) were used. Significant rise of antibody titer of 4-fold or greater during convalescence was seen only in the A-prime virus such as Fm_1 and the 53-Ben; the latter was isolated during the epidemic. Occasionally a concomitant 4-fold or greater rise of antibody titer against PR8 strain also occurred. However, no case had a significant antibody rise against the Lee strain of influenza B virus. Therefore only data of hemagglutination-inhibition tests on A-prime viruses are tabulated.

TABLE I. Results on Examination of 20 Patients during an Influenza A′ Epidemic.

Clinical diagnosis	Nasal smears*	Virus isolation	HI against A′, -fold rise
Influenza	+	+	32
"	+	—	64
"	—	+	8
Mononucleosis	—	—	0
Influenza	+	—	0
"	—	nd	4
"	+	—	4
"	+	+	8
"	+	—	16
"	+	+	8
"	—	+	32
"	+	—	4
"	—	—	64
"	+	—	128
"	—	—	8
"	+	—	32
Acute tonsillitis	—	—	2
Influenza	+	+	4
"	+	+	4
"	+	+	8

* Stained with fluorescent antibody solution against influenza A virus.

In only 13 cases nasal smears had definite specific fluorescent cells permitting a positive diagnosis of influenza A-prime infection (Table I). The diagnosis in 12 of these positive cases was confirmed by rise of antibody

TABLE II. Results of Rapid Diagnosis of Human Influenza A′ Infection by Fluorescent Antibody Staining of Nasal Smears.

Smears stained with fluorescent antibody	Positive virus recovery or 4-fold rise of HI	2-fold rise or no rise of HI	Total
Positive	12†	1	13
Negative	5	2*	7
Total	17	3	20

Positive smears: $12/17 = 71\%$

* 1 case of infectious mononucleosis. 1 case of acute tonsillitis.

† 6 cases with positive virus recovery.

TABLE III. Results of Rapid Diagnosis of Human Influenza B Infection by Fluorescent Antibody Staining of Nasal Smears.

Nasal smears from:	Smears stained with fluorescent antibody	Confirmed* influenza	Unconfirmed† influenza	Total
Normal saline nasal washings	Positive	10‡	1	11
	Negative	16	5	21
	Positive smears: 10/26 = 38%			
Nutrient broth nasal washings	Positive	1	0	1
	Negative	5	1	6
	Positive smears: 1/6 = 17%			
Swabbing of nasal turbinate	Positive	0	0	0
	Negative	4§	1	5
	Positive smears: 0			

* Confirmed by a 4-fold or greater rise of HI antibody titer during convalescence and/or virus isolation.

† 2-fold or no rise of HI antibody titer during convalescence.

‡ Including 2 cases of influenza A.

§ Including one case in which smears from swab method were negative but smears from saline nasal washings were positive.

during convalescence, and from 6 of them also an A-prime virus was recovered. Of 7 cases with negative nasal smears, 5 showed a rise in HI antibody, while 2 cases also had positive virus isolation Of the remaining 2 negative nasal smear cases, both were negative for virus isolation and one had a 2-fold rise of HI antibody. The clinical diagnoses of these 2 patients were infectious mononucleosis and acute tonsillitis.

Taking the 4-fold or greater HI antibody rise with or without positive virus isolation as the evidence for influenza infection, out of 17 confirmed cases, 12, or 71% showed positive smears (Table II).

Study of acute febrile illnesses. No influenza was encountered in the winter of 1953-4. However, 23 cases of various types of acute febrile illness or upper respiratory infections including 3 cases of clinical influenza were chosen at random for this study between late Nov. 1953 to Feb. 1954. In these 23 cases no rise of HI antibody against either influenza A or B viruses occurred. However, one patient with acute upper respiratory infection had a positive nasal smear when stained with influenza A' fluorescent antibody. Therefore, one false positive diagnosis was encountered among 23 non-influenzal cases.

Influenza B epidemic. In this study, a comparison of normal 0.85% saline solution and nutrient broth for nasal washings as well as a study of the use of cotton swabs for nasal smears was undertaken. The best results were obtained from nasal smears using normal saline for nasal washing (Table III); even so, only in 38% of these cases were virus-containing cells found in nasal smears under the fluorescence microscope. The nutrient broth nasal washings produced 1 positive out of 6 specimens. Nasal turbinate swabs from 4 cases were negative. Smears made from nutrient broth nasal washings or swabs had far fewer cells present than those prepared from saline washings. When there were too few cells in the smears, the chance of detecting cells containing influenza antigen was evidently greatly reduced.

Discussion. From the foregoing data, it is evident that a rapid diagnosis of human influenzal infection by means of fluorescein-labeled antibody staining of cells washed out from the nose is feasible. This method is simple and practical; and a diagnosis of influenza could be made on the day the specimens were collected. The shortcoming lies in a lower sensitivity than the hemagglutination-inhibition test. In attempting to explain the lower sensitivity of this rapid diagnostic method, it is possible that we have not been able to examine the patients early enough in their illness for nasal smears. From the experience in the study of experimental influenzal infection in ferrets, the period of

positive nasal smears is limited to 2 to 3 days of the comparatively short febrile period of the illness(3). No fluorescent cells were found in the smears before the fever started, probably because onset of fever usually coincides with desquamation of the infected ciliated epithelium into the nasal passage. The reason for the failure to detect cells containing viral antigens in nasal smears after subsidence of fever is not clear, since virus-recovery is still possible. Since patients in our study were healthy young male students, they usually did not seek medical care until they had had a fever and physical discomfort for about 24 to 48 hours, and were generally seen on the third day of their illness. This delay in the time of collection of the nasal washings may account for many of the negative smears. On the other hand, we do not know whether the pathogenesis of influenzal infection in human beings is similar to that in ferrets. It is likely that the desquamation of infected nasal epithelium in human patients may not be as extensive as in ferrets, for in the cases of human influenza uncomplicated by bacterial infections, the epithelium of the bronchioles is intact(9).

The fluorescent antibody solutions used in these studies were not made from current epidemic strains of influenza virus. However, they could be used because of known cross reactions in fluorescent staining among different strains of influenza A virus due to the presence of a common soluble antigen(10,11). Presumably the same is true for influenza B virus. The significant difference between sensitivity of influenza A epidemic study (71% positive) and influenza B epidemic study (38% positive) could be due to a less potent fluorescent conjugate prepared from the Lee strain of influenza B virus or to a milder B infection with less epithelial desquamation.

As yet, there is no perfect diagnostic method for influenzal infections. Even serological tests or isolation of virus do not detect 100% of the infections. The present method is less sensitive than the hemagglutination-inhibition. It is hoped that sensitivity may be increased with more experience. However, if therapeutic agents for viral infections ever become available, retrospective diagnostic tests might become impractical and a rapid diagnosis of the disease becomes desirable.

Summary. A specific rapid diagnosis of human influenzal infection by staining smears of nasal washings with fluorescein-labeled antibody solution is described. This method was applied to 20 patients during an outbreak of influenza A-prime infections, to 44 cases during an outbreak of influenza B, and to 23 cases of various acute febrile or respiratory illnesses. Taking a rise of 4-fold or greater in titer of hemagglutination-inhibition (HI) antibody during convalescence, or the recovery of virus from human nasal washings as diagnostic criteria for influenzal infection, the use of fluorescent antibody had a sensitivity of 71% in an influenza A-prime and 38% in an influenza B epidemic One false positive diagnosis was encountered among 23 non-influenzal infections. The use of labeled antibody on smears of nasal washings is less sensitive than the HI test, but a positive diagnosis could be made on the day the specimens were collected, while the HI test requires a delay of 10 to 14 days for collection of convalescent serum.

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