Laboratory Diagnosis of Asian Influenza

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MODERN STUDIES of influenza are dependent upon information obtained in the laboratory. Clinical impressions and epidemiological observations may suggest influenza. But until virus isolation or antigenic experience has been demonstrated, an etiological diagnosis cannot be made since many other agents can produce an influenza-like syndrome (*1*).

The role of the laboratory in defining and following the spread of an infectious agent has never been more dramatically shown than in the present epidemic of Asian influenza. From the time of the earliest antigenic analyses (*2, 3*) it was predicted that this new variant of the influenza virus would spread rapidly and that epidemics would occur throughout the world. It is the purpose of this report to describe the laboratory facilities which are now engaged in a large-scale operation aimed at providing definitive information about influenza. Some of the results obtained during these activities will also be presented.

Laboratory Network

The Laboratory Branch of the Communicable Disease Center, Public Health Service, began to intensify its work with influenza in 1955, and at that time, the Virus and Rickettsia Section accepted responsibility for an influenza center of the World Health Organization. In 1956, the CDC Virus and Rickettsia Section established a Respiratory Disease Unit at Montgomery, Ala., which was concerned with studies of influenza and several other viruses from the respiratory tract. This unit was designated by the World Health Organization to be the International Influenza Center for the Americas. In February 1957 this center sponsored travel for its director to the World Influenza Center in London for an exchange of concepts and discussions of problems.

The WHO influenza program consists of a worldwide network of collaborating laboratories and observers that report to the centers concerning the occurrence of influenza and forward virus isolates for antigenic analyses. This program was initiated in the United States in 1948 with approximately 40 laboratories participating.

Several State and Territorial health department laboratories have collaborated with the program during the past 9 years, and in the past few months, with help from CDC, many more of these laboratories have been able to participate in the program. There are now 45 health departments actively supporting the Public Health Service and WHO investigative projects. Currently a total of 135 laboratories are members of the American network. In the United States many of these are located at universities, institutes, and medically oriented organizations as shown below.

United States	115
State and Territorial health departments	45
Universities	39
Institutes	11
Hospitals	8
Municipal	6
Armed Forces	3
Drug companies	3
Canada	3
Central America	4
South America	13

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Laboratory Support Program

The pattern of providing financial and other types of assistance to laboratories, principally those of official health agencies, as later discussed, had been established earlier in the poliomyelitis diagnostic field. Anticipating a need for specific diagnosis of influenza, in order that accurate estimates of attack rates could be obtained, a basically similar program was planned by the Communicable Disease Center. This as executed consists of reimbursing official health agency laboratories for contractual services, which include establishing and maintaining facilities for isolating and typing influenza virus, maintaining serologic procedures, and reporting results of laboratory study of cases to the Center.

Certain academic or institutional laboratories have essentially similar types of contracts to provide this service to States, or to serve in a resource capacity in the evaluation of materials, procedures, or skills.

The facilities thus established are intended to perform functions expected to provide better intelligence on influenza than might otherwise be expected. The services of the laboratories will include examination of clinical materials from index cases of respiratory diseases in order that local influenza outbreaks may be early recognized. Since much influenza-like disease is prevalent, the workload involved in the early detection of influenza has been substantial. Additionally, the laboratories participating in the program are directing special efforts toward obtaining isolates, either viral or bacterial, from persons with severe influenza infections, and similar material from fatal cases that come to autopsy. All cooperating laboratories are participating in a technical evaluation of procedures and skills so that appraisal of the value of the different diagnostic techniques can be made and so that some estimate of the range in results of tests in the various laboratories can be determined.

Earlier Observations

Variations among type A influenza viruses have been studied for many years but significant antigenic differences and relationships have been most clearly demonstrated by field trials of vaccine. In the laboratory, results of antigenic comparisons of the different strains with cross-tests utilizing various antiserums have provided detailed information about shared antigenic components. It has also been possible to show that periods of prevalence for virus strains with particular major antigens are reflected in patterns of antibody titers in human serums from various age groups. These developments have been reviewed elsewhere (*4*). It was evident from these past studies that a new set of type A variants of the influenza virus could be expected.

One of the lessons learned during the past 15 years was that influenza vaccines could be relied upon to be effective in reducing the incidence of the disease only when the vaccine formula included strains representative of the prevalent set of viruses. Recognizing this, the World Health Organization established strain study centers with many strategically located surveillance laboratories with the view toward rapid identification of virus isolates from each new outbreak. The viruses thus obtained in Singapore, Formosa, and Japan during May 1957 were strikingly different from previous type A strains. The incidence of antibody against these viruses in human serums was extremely low. The expected new set of viruses had appeared and promised to spread uninhibited throughout the world population. Anticipating this, the WHO program participants and responsible military and Public Health Service officials were alerted, and within a week prototype strains were sent to vaccine manufacturers for pilot studies and to the collaborating laboratories so that each could become familiar with the characteristics of the new agent.

Diagnostic Reagent Program

Although many laboratories are capable of preparing the antigens and antiserums necessary for the diagnosis of influenza infections, a majority rely upon a central source for these materials. Since it was realized that early detection of influenza would depend upon the general availability of these reagents, in June 1957 approximately 100 laboratories were sent 15 ml. each of viral antigen prepared with the A/Jap/305/57, the A/Denver/1/57, and the B/GL/1739/54 strains. The last two strains are representative of recent variants of types

A and B. Antiserums prepared in chickens with each of these viruses were also distributed to laboratories in 5.0-ml. volumes. By the end of October, more than 30,000 ml. of antigen and 10,000 ml. of antiserum had been mailed to more than 300 laboratories.

Results of tests in July made it clear that the antigen prepared with the Japan 305 strain transferred serially in embryonated eggs was useful only in complement fixation (CF) tests and not in the hemagglutination inhibition (HI) method. Serum from patients convalescent from Asian influenza did not readily inhibit the hemagglutination of chicken erythrocytes by this virus. Meanwhile the virus had been instilled into the respiratory tract of ferrets, causing death, and infective suspensions of the lung were transferred serially in mice and later in eggs. This "animal-line" virus was much more sensitive to antibody in HI tests. Although mechanisms concerned in converting influenza strains from an antibody-insensitive phase to the more reactive phase are not understood, the phenomenon is well known and has been witnessed in many laboratories.

Another technical matter had to be dealt with, however, before the animal-transferred line of virus could be utilized. In addition to becoming more reactive with antibody, this line was also inhibited to a high degree by nonantibody substances in human and animal serums. The nonspecific inhibitors could not be digested with trypsin, but overnight treatment with solutions of periodate completely destroyed the troublesome inhibitors without markedly reducing specific antibody titers.

Therefore, beginning in August the antigen supplied to laboratories for the detection of Asian influenza antibody response has been that prepared with the egg-ferret-mouse-egg line (EFME). Directions for use of the antigen have been supplied. The viable seed for this Japan 305 variant and the other strains have also been distributed, and other materials, including human serums and normal allantoic fluids for control purposes, have been furnished upon request.

Information and Training Activities

Training in laboratory techniques in use at the International Influenza Center was provided in a 2-day workshop at the end of September. A class of 62 State and Federal technicians and directors was instructed in procedures to be followed in the isolation of influenza viruses from suspected materials. Standard methods for performance of the CF and HI tests were discussed and incorporated in laboratory exercises. Techniques for production of diagnostic reagents were outlined and considerable time was spent in discussing the interpretations of data.

The International Influenza Center met the problem of communicating pertinent recent information, or methodology, to laboratories by periodic newsletters and by collaboration with the CDC Influenza Surveillance Unit in the publication of a weekly influenza report.

Characteristics of Virus Isolates

The nature of this report does not allow a detailed description of observations made about the viruses isolated in the past few months. However, the following statements may serve to summarize the findings:

- All type A isolates obtained from various parts of the world since June are closely related antigenically to those sent from Singapore. The Asian set apparently has replaced the A′ or FM family. Approximately 200 type A viruses have been forwarded to the International Influenza Center during this time, and all have been readily typed as the Asian variety. Three type B strains have been received, and these are very similar to the B/GL/54 strain contained in polyvalent influenza vaccines.
- The rate of successful virus isolations has been extremely variable (from 0 to 100 percent) and is obviously dependent upon several prelaboratory factors, such as care taken in selection of patients, procedures followed in specimen collection, and transfer of specimens to the laboratories. In the laboratory, proper storage and treatment of specimens are necessary. Although the amniotic inoculation of 11-day embryonated eggs continues as the method of choice, many investigators have found monkey kidney culture useful (*5*). It is clear that amniotic fluids harvested from eggs should be tested with suspensions of guinea pig erythrocytes to detect the presence of hemagglutinating

virus. Often fluids shown to be negative with chicken erythrocytes were definitely positive with cells from guinea pigs. The value of subinoculating negative pools of amniotic fluids into another group of eggs has also been demonstrated. In several laboratories over 50 percent of the successful isolations were noted in this manner.

• Many of the positive fluids contained only low concentrations of hemagglutinating virus so that from 2 to 4 additional transfers were necessary before good titers could be obtained. Several strains have been obtained, however, which routinely reach titers of 1 : 800 or greater with 0.5 percent suspensions of chicken erythrocytes. Parallel tests of human serums with the Japan 305 strain and some of the more recent isolates have been carried out. One of these strains which yields higher titers may be substituted in the near future for the Japan 305 as the diagnostic antigen.

Serologic Diagnosis

Not all strains of influenza virus are equally sensitive to antibody in the HI or neutralization of infectivity tests. With the use of some variants the antibody response to infection in humans or animals cannot be demonstrated except by CF tests. On the other hand, many isolates have been studied which are as efficient as the Japan 305 EFME line supplied by the International Influenza Center. Mogabgab has found that fluids from infected monkey kidney cultures may also prove equally satisfactory sources of antigen (personal communication).

Many laboratories have reported diagnostic increases in antibody titers measured with A/Denver/1/57 or older type A influenza viruses in CF or HI tests although the isolates cultured from these patients were in the Asian set. These results are not surprising. The lack of specificity in antibody response to influenza infection, especially with CF tests, has been known for years. Repeated observations of antibody increases measurable in HI tests with strains of influenza virus no longer prevalent have led to the development of the "doctrine of original antigenic sin" (*6*). Although the mechanisms concerned remain poorly defined, it is clear from results of vaccination experiments and serum absorption tests that antibody response to related antigens is often conditioned by previous antigenic experience. In this epidemic of influenza some patients have responded by producing increased antibody titers against the older virus strains with no detectable production of antibody against the Asian set. Viruses obtained from these cases were always of the Asian variety. Therefore, serologic diagnosis cannot be relied upon as a means of defining the set of the infecting agent but only as defining the broader immunological type.

Comparison of number of antibody responses measurable by complement fixation or hemagglutination inhibition tests

Antibody response	Number	Percent
Total cases diagnosed [1]	123	100. 0
CF positive	94	76. 4
HI positive	76	61. 8
CF positive, HI negative	47	38. 2
CF negative, HI positive	29	23. 6
CF positive, HI positive	47	38. 2

[1] Fourfold or greater increase in antibody titer measured with either test.

The observations discussed above appear to lead to the logical conclusion that not much is to be gained by employing the HI test. The CF test will define the type of infection. The series of results compiled in the table, however, make it clear that both tests must be employed to effect a maximum of serologic diagnoses. Although the CF procedure was more often the more sensitive, many cases could not be diagnosed without the use of the HI method. Often high titers were found in the acute phase serum with the CF antigens while HI antibody was very low. In these cases the HI test is most useful. In a surprisingly low percentage of cases, only 38.2 percent, could the diagnostic rise be demonstrated by both tests.

Discussion

Legions of contributors to the store of knowledge about influenza have joined in an effort to study the characteristics of pandemic influenza. Although it is obviously too early at this time to define what has been learned, many laboratory

observations made during the past several years have been dramatically reconfirmed. The central role of the laboratory in providing etiological diagnosis of sporadic outbreaks and epidemics of influenza has been emphasized. Spread from country to country and within geographic areas has been carefully observed. There have been discussions in the past as to whether new variants appear simultaneously in several areas or the spread is from a single source. In the case of Asian influenza, at least, there can be no argument; the new variant arose in China and spread from that point.

Great interest has been focused on the question of whether strains of both the FM and Asian sets of A influenza would circulate. From June through November 1957 viruses isolated in great networks of laboratories all over the world have been of the Asian variety only. The greatest probability is that the older A sets will not be seen again in the near future. If the antigenic variations continue the course set during the past 24 years, we can expect modified forms of the Singapore viruses to appear and circulate during the next 10 years. The great shifts in antigenic composition of influenza viruses have occurred at least three times in our history at intervals of about a decade. Meanwhile, we can expect protection from influenza by vaccination or other antigenic experience with any of the Asian set of influenza virus strains.

Antibody response is often not strain specific in man because of previous conditioning by experiences with other variants of the immunological type. When epidemiological information is desired about more precise antigenic definition of the etiological agent, virus isolates must be examined. Although the serologic diagnosis of influenza is more frequently afforded by CF technique, a significant number of cases will be missed unless the paired serums are also tested by HI tests.

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Berlin Epidemic, 1889

"Professor Virchow has recovered from his attack of influenza; several members of the Imperial family have also been attacked . . . The *National Zeitung* is censuring those who spread pessimistic theories about the epidemic. . . . Official data of the number of cases of influenza here have not been made. It is known . . . that but few families have escaped the plague, and that a third of the population has been ill with it. The medical press refutes the theory that there is any connection between the influenza plague and cholera, although it is known to be a fact that cholera has repeatedly appeared after an influenza epidemic."

—Cable report from Der Deutscher Correspondent, Baltimore, Md., Public Health Reports, December 14, 1889.