JOURNAL OF CLINICAL MICROBIOLOGY, July 1977, p. 19-22
Vol. 6, No. 1

Printed in U.S.A.

Maintenance of Viability and Comparison of Identification Methods for Influenza and Other Respiratory Viruses of Humans

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Received for publication 14 February 1977

A comparison of Hanks balanced salt solution, veal infusion broth (VIB), and charcoal viral transport medium for maintaining viability of type A influenza virus indicated approximately equal survival of virus on all three media at −70 and 4°C, whereas at 25°C virus survived best in VIB. VIB supplemented with bovine serum albumin was used as transport medium in a community-wide surveillance of febrile respiratory disease for influenza viruses. Unfrozen throat swab specimens were placed in VIB and stored at 4°C for up to 5 days without effect on isolation frequencies of either type A or type B influenza virus or type 1 or type 3 parainfluenza virus. Comparison of indirect immunofluorescence with hemadsorption for detection of type A influenza virus in rhesus monkey kidney cultures revealed a requirement for at least five fluorescing cells to eliminate false positive indirect immunofluorescence tests and at least 3 days of incubation to eliminate false negative tests when compared with hemadsorption at later times. Detection frequencies for the two methods after 2 and 3 days of incubation were not significantly different.

Viral diagnosis of respiratory disease has been restricted by the view that many of the causative agents are relatively labile and that rapid identification procedures are unavailable.

During the winter season of 1974–1975, a physician-based, community-wide surveillance system for the virological monitoring of respiratory tract illnesses was instituted in Houston, Tex. The principal goal of the program was to develop epidemiological data for the influenza viruses. Since inception of the program, outbreaks of type A and B influenza and type 1 and 3 parainfluenza virus infections have been documented. An analysis of relative virus isolation frequencies showed that these agents could be maintained at 4°C without significant loss in infectivity for an extended time interval. In addition, a comparison of the hemadsorption (Had) and immunofluorescent-antibody (IFA) techniques for early detection of type A influenza virus reaffirmed the utility of both techniques for this purpose. The data leading to these results are presented in this report.

MATERIALS AND METHODS

Virus isolation procedures. Throat swab specimens were obtained on a daily basis from patients of all ages presenting with a febrile respiratory illness to a cooperating family physician or to a neighborhood clinic supported by the Harris County Hospital District. After collection, the swab was placed in 3 ml of medium consisting of veal infusion broth (VIB) and bovine serum albumin (BSA) in a final concentration of 0.5%. Delivery intervals to the laboratory for processing of specimens ranged from the day of collection to 5 days later. Specimens were assayed for hemadsorbing viruses by inoculation of 0.4 ml of undiluted fluid into a single conventional rhesus monkey kidney cell culture. Presence of virus was determined routinely on days 5 and 10 by the Had technique (7). During the time period when an A/Port Chalmers-like (H3N2) virus was prevalent in the Houston community, a portion of the specimen collection was inoculated concomitantly into Leighton tubes containing a cover slip with the same type of cell monolayer. In these cultures, viral replication was detected by the IFA test described below. The maintenance medium for both types of cell cultures consisted of 1.5 ml of Eagle minimal essential medium containing antibiotics.

Identification procedure. For each conventional rhesus monkey kidney cell culture exhibiting Had, cell suspensions were prepared and placed in 50-μl volumes to five separately spaced areas on glass slides. Slide preparations and cover slip monolayers from Leighton tubes were air-dried and fixed in acetone (the latter step is no longer used) before evaluation by the indirect IFA test (4).

Hyperimmune antisera to A/England/72 (H3N2), B/Hong Kong/72, and type 1, 2, and 3 parainfluenza

viruses prepared in guinea pigs were inactivated (56°C for 30 min) and absorbed with a 15% suspension of baby beef brain powder (8). The highest dilution of each antiserum producing a 4+ staining pattern and negative staining of uninfected control cultures was used. Monospecificity of the reagents was established in reciprocal cross-IFA tests.

Antiserum to type A and B influenza viruses were placed separately on a single fixed tissue area of glass slides or sectioned cover slips. These areas were thoroughly washed with phosphate-buffered saline (PBS) and reacted with fluorescein-conjugated rabbit anti-guinea pig 7S immunoglobulin G (Hyland) diluted 1:30 and a 1:20 solution of rhodamine in PBS. After a further incubation at 37°C for 30 min, the cells were thoroughly washed with PBS, rinsed quickly with distilled water, air-dried, and coated with buffered glycerol. Slide preparations negative for the influenza viruses were tested in the same manner by reacting untreated cell areas with antisera to the parainfluenza viruses.

RESULTS

Effect of various conditions on virus survival. An initial study using type A influenza virus was done to establish an acceptable routine for collecting patient specimens. A 1:100 dilution of a pool of throat swab specimens obtained from persons infected with A/Port Chalmers-like virus previously stored for approximately 1 month was made in fresh VIB without BSA, Hanks balanced salt solution, and charcoal viral transport medium (CVTM) (6). At periodic time intervals, serial 10-fold dilutions were made in Eagle minimal essential medium and assayed for infectivity, using the same lot of tissue cultures. The mean of two independent titrations for each medium and storage temperature is presented in Fig. 1. No significant loss in viability of infectious virus was evident in any of the media stored for 7 days at −70°C. Although not shown, infectivity was maintained for 14 days at this temperature. The results obtained at 4°C showed that virus survival could be maintained satisfactorily for 5 days. A marked difference in the relative rates of viral decay was noted at 25°C. By day 3, less than 10% of the virus stored in salt solution and CVTM remained viable. In VIB, a similar reduction in virus titers was evident by day 5.

Virus isolation frequencies of patient specimens. On the basis of the above results, throat swab specimens collected in the surveillance system were placed in VIB with BSA after collection and stored at 4°C for up to 5 days. A determining factor for instituting this procedure was that economic considerations precluded installation of −70°C storage facilities at the multiple surveillance sites. In addition, although not tested for effect on stability of virus, BSA was added because of the report that optimal isolation of parainfluenza viruses is obtained with VIB containing BSA (1).

During an A/Port Chalmers-like (H3N2) epidemic in Houston in 1974–1975, 1,189 specimens were obtained from persons with a febrile respiratory disease. The relative isolation frequencies according to the number of storage days before initiating virus isolation studies are presented in Table 1. Except for 73 specimens, the others were held at 4°C for 1 or more days. The isolation rates for the holding period ranged from 34 to 47%. The differences, however, were not statistically significant ($P > 0.05$). All but 1.4% of the virus-positive specimens were detected when tested for Had at 4 days after inoculation in cell cultures; the remaining number were positive after a further 5-day incubation period.

Similar analyses of isolation frequencies for type B influenza virus and type 1 and 3 parainfluenza viruses are shown in Tables 2 and 3. Data for the former represent results obtained from an outbreak of 7 weeks' duration in 1976, and for the latter, a 35-week surveillance period in 1974–1975. Although there were some differences in the frequency of positive specimens for

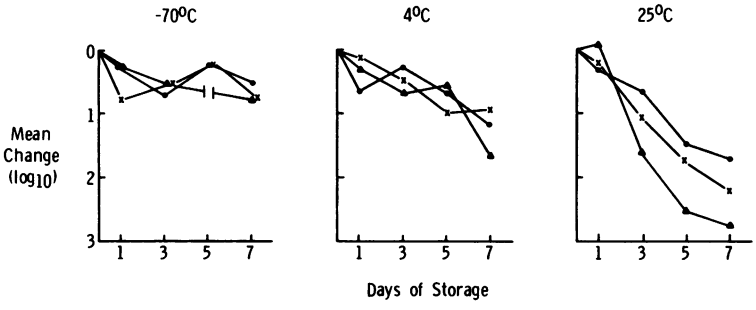

FIG. 1. *Infectivity decay curves for type A/Port Chalmers-like (H3N2) virus at different storage temperatures in (●) veal infusion broth, (×) Hanks balanced salt solution, and (▲) charcoal viral transport media. Concentration of virus at time zero was 3.7 $\log_{10}$ per ml.*

TABLE 1. *Type A influenza virus isolation rates from patient specimens after storage at 4°C*

Days of storage	No. of specimens tested	No. of positive specimens	% Positive
0	73	25	34
1	432	186	43
2	261	103	39
3	300	111	37
4	123	58	47
5	73	28	38

TABLE 2. *Type B influenza virus isolation rates from patient specimens after storage at 4°C*

Days of storage	No. of specimens tested	No. of positive specimens	% Positive
0	303	4	1.3
1	235	6	2.6
2	101	6	5.9
3	88	2	2.3
4	103	6	5.8
5	163	4	2.5

TABLE 3. *Parainfluenza (para) type 1 and 3 virus isolation rates from patient specimens after storage at 4°C*

Days of storage	No. of specimens tested	No. (%) of positive specimens	
		Para 1	Para 3
0	866	10 (1.1)	24 (2.7)
1	1,469	18 (1.2)	32 (2.1)
2	633	6 (0.9)	16 (2.5)
3	650	1 (0.1)	14 (2.1)
4	357	9 (2.5)	9 (2.5)
5	216	1 (0.4)	3 (1.3)

the storage period of the three viruses, the number of virus recoveries did not vary significantly (in each instance, $P > 0.05$). Of particular interest was the relatively low order of virus isolations of type B influenza virus when it was present in the community.

Detection of type A influenza virus by the Had and IFA methods. During the A/Port Chalmers-like epidemic, 303 of the patient specimens were inoculated concomitantly into Leighton tubes containing cover slips with the same type of monolayers as the conventional type of culture. The relative efficiency of demonstrating virus-positive cultures was compared by determining isolation rates obtained in IFA detection tests after a 2-day incubation in Leighton tube cultures to the 5- and 10-day Had method (Table 4). As shown, over 90% of specimens eventually positive by Had were detected by IFA at 48 h. However, a small proportion of specimens thought positive by IFA were negative by Had. Nineteen of the 20 specimens exhibiting lack of agreement on the two tests were available for retesting. The original Had result was confirmed. Repeat testing of the apparent false positive and false negative IFA tests with performance of the IFA test after 72 h of incubation provided complete agreement with the Had result.

To establish criteria that would effectively eliminate false positive IFA tests, 55 specimens were retested by both methods. When the criterion of staining of at least five cells, scattered or in one or two foci, were used, no false positive IFA tests occurred. Shown in Table 5 are the isolation frequencies by each method for the 43 specimens in this test exhibiting Had by day 5. Although the frequency of positive specimens on days 2 and 3 for IFA was higher than for Had, the differences were not statistically significant ($\chi^2 = 2.87$, $P = 0.09$).

DISCUSSION

It is highly desirable to perform viral diagnostic tests within a relatively short time period after collection of clinical specimens. However, in the conduct of daily virological surveillance on a community-wide level, this is not practical because of operational and economic considerations. The data in this report provide information on the isolation frequencies of type A and B influenza and type 1 and 3 parainflu-

TABLE 4. *Comparison of the IFA method at 48 h with the Had procedure at 5 and 10 days for detection of type A influenza virus*[a]

Influenza virus isolated (Had)		Influenza virus not isolated (Had)	
IFA positive	IFA negative	IFA positive	IFA negative
93 (30.7)[b]	9 (3.0)	11 (3.6)	190 (62.7)

[a] Percent co-positive: 91; percent co-negative: 95; overall agreement: 93%.

[b] Numbers in parentheses are percentages.

TABLE 5. *Relative isolation frequencies by the IFA and Had methods according to incubation periods*

Detection method	Specimens positive on indicated day[a]		
	2	3	5
Had	32/43 (74)	38/43 (84)	43/43 (100)
IFA	33/43 (77)	42/43 (98)	ND

[a] Numerator indicates the number of positive specimens, and denominator indicates the number of specimens tested. Numbers in parentheses are percentages. ND, Not done.

enza viruses after storage of patient specimens at 4°C for varying periods of time. A principal finding of the study was that survival of virus was not adversely affected by holding specimens for as long as 5 days. For each type of virus, the rate of isolations as detected by the Had test for each duration of storage was relatively uniform. The similarity in isolation frequencies may have occurred because the specimens were collected from individuals with a febrile respiratory disease seeking medical attention who probably contained high concentrations of virus. This view is supported by reports that the quantity of virus shed is directly related to the severity of influenzal illness (2, 5). Based on the results of the virus survival experiment, sampling of mild or asymptomatic cases of influenza might require inoculation of specimens within 48 h after collection or storage at −70°C until diagnostic tests can be performed in order to overcome loss of infectivity.

Of the two detection systems, the IFA using Leighton tube cover slips tended to provide the earliest identification of type A influenza virus. However, although a high proportion of the specimens could be identified after a 48-h incubation period, the isolation frequency equal to that obtained by the Had method was only observed after 72 h. These results are in general agreement with those reported by others (3). For surveillance studies, the preparation of slides for IFA tests with cell cultures that were Had positive provided a relatively inexpensive procedure for increasing the number of identifications that could be performed using a single-cell culture tube.

Serological surveys and limited virological studies are generally the principal means by which epidemiological data are obtained on respiratory viral diseases in humans. In this report, procedures are described that should be of value for improving virological surveillance of these infections on a community-wide basis.

ACKNOWLEDGMENTS

This work was supported by Public Health Service contracts AI 32506 and AI 42528 from the National Institute of Allergy and Infectious Diseases and by Contract 9-13139 from the National Aeronautics and Space Administration.

We wish to gratefully acknowledge the interest and cooperation of the following groups and persons in obtaining specimens for this study: the Department of Community Medicine, Baylor College of Medicine, and the Neighborhood Clinics of the Harris County Hospital District; the Ben Taub Hospital; and family physicians in the Houston community.

We wish to thank Cynthia Brinkley and Francoise Brasier for their excellent technical assistance.

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