Direct Identification of Influenza Virus in Throat Washings by Means of Concentration and Micro-Assay. (29814)

LENORE R. PEIZER (Introduced by Morris Schaeffer)
Bureau of Laboratories, Department of Health, New York City

The routine laboratory diagnosis of influenza from throat washings (T.W.) consists of passages of small portions of specimens through the amnions of fertile eggs. At least 2 serial amniotic passages are required to adapt influenza virus to fertile eggs and replicate sufficient amounts for its detection by hemagglutination (TA) of chicken or guinea pig red blood cells (CRC, GPRC). This report describes a procedure of concentration and of partial purification of influenza virus from throat washings. HA and HI tests were performed directly on concentrates of 6 known influenza A_2 positive, 2 known negative and 112 unknown throat washings. Final results were obtained 24-36 hours after the specimens reached the laboratory.

Materials and methods. Specimens: Sterile veal infusion broth or a more palatable skim milk preparation were used to collect TW. Skim milk was made by using instant Starlac* prepared in distilled water according to labelled instructions and autoclaved at 121°C for 15 minutes. Both preparations were dispensed in 12 ml quantities in 100 ml wide mouth jars for use. To be satisfactory, specimens had to arrive in the laboratory within 2 hours after collection or they had to be frozen immediately and sent to the laboratory, as soon as possible, packed in dry ice to prevent thawing in transit.

Concentration of specimens: In the laboratory, 3 ml of each TW were removed for routine egg inoculations. The remaining 9 ml of veal infusion TW were centrifuged at 5,000 rpm for 20 minutes in the No. 40 rotor of the model L Spinco centrifuge. The supernate was decanted into a stainless steel centrifuge tube and mixed with sufficient 1% gelatin in broth to obtain a final concentration of 0.1%(1). The specimen was centrifuged in the Spinco for 2 hours at 39,000 rpm with the brake off. The supernate was decanted and discarded. The sediment was resuspended in 1 ml of veal infusion broth and this partially purified concentrate was used for identification of influenza virus by means of a micro-test to be described.

The skim-milk specimens were more difficult to process and to test for virus than those taken in broth. For concentration additional centrifugation and elution of virus were required. The procedure was as follows: 1. Each specimen was centrifuged 10 minutes at 1000 rpm, the supernate decanted and saved. 2. The supernate was then centrifuged in the Spinco centrifuge, model L rotor No. 40 at 10,000 rpm for 1 hour. 3. Each sediment (pH 6.0-6.4) was completely emulsified in 6 ml of buffered saline (pH 7.3) at 4°C, packed in an ice bath and shaken well for 10-15 minutes. 4. The emulsions were then centrifuged in the Spinco centrifuge at 10,000 rpm for 45 minutes. The clear supernates were decanted from the plastic centrifuge tubes into stainless steel centrifuge tubes. 5. To each 4.5 ml of clear supernate was added 0.5 ml of 1% gelatin in saline. 6. These mixtures were spun down in the Spinco centrifuge at 39,000 rpm for 2 hours with the brake off. The supernates were decanted and discarded. 7. The sediment emulsified in 0.5 ml of physiological saline was used for identification of influenza virus by means of micro-HA and micro-HI spot tests to be described.

A schematic representation of the above procedure is shown in Fig. 1.

HA and HI micro-tests. Chicken red cells (CRC): CRC collected in Alsever's solution were washed 3 times with physiological saline. The cells were centrifuged at 1500 rpm for 10 minutes in a 50 ml graduated conical centrifuge tube, and then resuspended in 10 volumes of buffered saline. This 10% suspension of CRC was used to prepare the 0.2% of cells used in the micro-tests.

Receptacle for test: Since very small quantities of reagents were available, the Takatsy

* Borden Co., Bronx, N.Y.C.

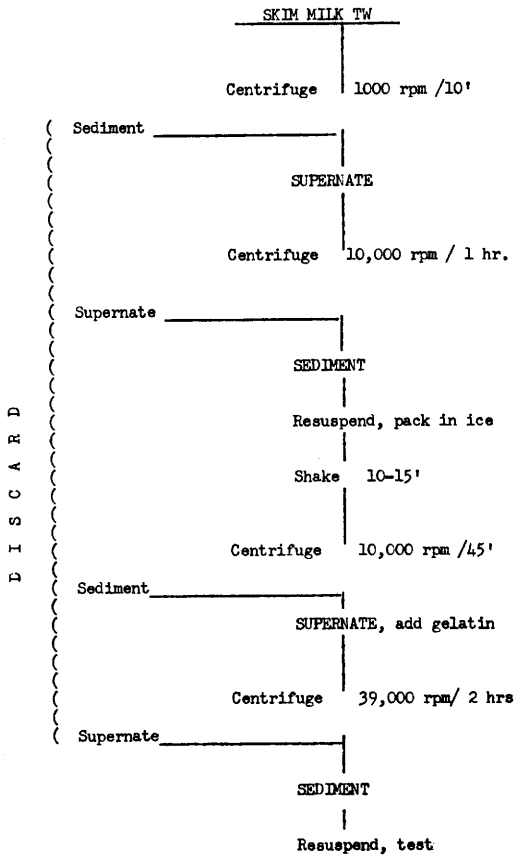

FIG. 1. Preparation of skim milk TW for direct micro HA and HI tests.

plastic plate† (2) was used. Very small quantities of all reagents were required and no distortion of CRC formations was evident.

Antisera: Roosters were immunized by a single simultaneous massive intraperitoneal and intravenous injection of live influenza virus. They were exsanguinated 3 weeks after infection. The sera were inactivated at 56°C for ½ hour, and treated as follows: Two volumes of 0.09 M KIO_4 in distilled water were added to one volume of serum and incubated overnight at 4°C. After incubation, 2 volumes of glycerine (1%) in buffered saline were added and the mixture left at room temperature for ½ hour in order for the glycerine to inactivate the KIO_4. The immune serum, now diluted 5-fold was centrifuged at 2000 rpm for 10 minutes to clarify it.

HA and HI tests on broth TW concentrates: Dilutions of each throat washing concentrate derived from veal infusion specimens were prepared in broth as follows: Undiluted, 1:10, 1:20, 1:40, 1:80, 1:160. Four hundredths (0.04) ml of each dilution was delivered into a separate well of a Takatsy plate by means of a 1 ml tuberculin syringe and 22-gauge needle with sawed off point (one drop equals 0.01 ml). By delivering the higher dilutions first, it was possible to use one syringe and needle for each specimen. Four hundredths (0.04) ml of 0.2% CRC in saline was added to the concentrate dilutions in the wells. Chicken red cell controls consisting of 0.04 ml of a broth-gelatin concentrate plus 0.04 ml of 0.2% CRC were prepared in duplicate. The Test was incubated in the refrigerator for 1 hour. The highest dilution showing complete hemagglutination was considered to contain one unit of virus.

The HI Test on HA positive veal infusion concentrates was performed as follows: Starting with 1:8 dilution (5 vol of inactivated serum plus 3 vol of saline) of centrifuged inactivated rooster immune serum in buffered saline, 2-fold dilutions of the antiserum were prepared up to a final dilution of 1:512. Two hundredths (0.02) ml of each dilution of immune serum was delivered into wells of a Takatsy plate by using the technique as previously described (1 drop, 0.01 ml). To each serum dilution were added 0.02 ml (2 drops) of concentrate diluted to contain 4 units of virus as determined by the HA test. The plate was rotated to mix the reagents and incubated in the refrigerator for 1 hour. After incubation, 0.04 ml of 0.2% CRC was added and the test reincubated at 4°C for 1 hour.

Immune sera used in the HI tests were types A_2, FM_1 and B/GL. Virus controls for the HI test consisted of 4 units of virus in 0.02 ml of concentrate dilution plus 0.02 ml of broth plus 0.04 ml of 0.2% CRC added after incubation of virus and broth. Immune serum controls consisted of 0.02 ml of 1:8 dilution of immune serum plus 0.02 ml of broth plus (after incubation) 0.04 ml of 0.2% CRC. Chicken red cell controls were the same as in the hemagglutination test. All controls were set up in duplicate. Known

† Cooke Engineering Co., Alexandria, Va.

TABLE I. Ratio of Infectivity to Hemagglutination Titer for Throat Washings and First Passage Amniotic Fluids.

Specimen No.	Throat washings						First passage amniotic fluids from eggs inoculated with throat washings					
	Crude			Concentrated			Crude			Concentrated		
	I	HA	I/HA	I	HA	I/HA	I	HA	I/HA	I	HA	I/HA
1	<1*	<1	?†	10	30	.3	150	<10	>15	300	15	20
2	10	1	10	25	60	.4	300	50	6	550	150	3
3	<1	<1	?	1	30	.03	75	<10	> 7.5	250	75	3
4	<1	<1	?	10	15	.7	75	<10	> 7.5	150	< 10	>15
5	<1	<1	?	25	60	.4	250	15	17	450	75	6
6	<1	<1	?	10	15	.7	150	<10	>15	300	< 10	>30

* I = infectivity titers expressed as EID_{50} for 0.2 ml inoculum.
† ? = indeterminate.

negative and positive throat washing concentrates were included in HA and HI tests of all unknown specimens and were treated in like manner as the unknown concentrates.

HA and HI tests on concentrates derived from milk specimens: These were spot tests and were performed with undiluted concentrates as follows: 1. To 0.03 ml of specimen concentrate in a Takatsy well, 0.03 ml of saline and 0.06 ml of 0.2% CRC in saline were added. 2. This was mixed well and incubated in the refrigerator at 4°C for at least 2 hours or overnight. 3. After incubation the test was examined for hemagglutination. 4. CRC controls, consisting of 0.03 ml of skim-milk concentrate, (not TW) 0.03 ml of saline and 0.06 ml of 0.2% CRC in saline, were treated in the same manner as skim-milk specimens.

The HI test on skim-milk concentrates was a spot test performed on undiluted concentrate and (1:8) dilution of inactivated immune serum. The types of influenza antisera used were A_2 and B/GL. The procedure was as follows: 1. 0.03 ml of concentrate was delivered into a Takatsy well. This was mixed well with 0.03 ml of 1:8 dilution of immune serum. 2. The mixture was incubated in the refrigerator for 2 hours. 3. Then 0.06 ml of 0.2% CRC was added and mixed well. 4. The test was incubated in the refrigerator at least 2 hours or overnight. 5. Serum controls were included in the HI test. 6. The test was examined for compact button masses (inhibition of agglutination) and for hemagglutination of CRC.

HA and HI spot tests on the 57 concentrates derived from skim-milk specimens were performed simultaneously on all specimens to obtain a rapid diagnosis.

Results. The results of a pilot study on TW concentration and direct identification by micro-tests of 6 known influenza A_2 positive TW are summarized in Table I. The concentrates consistently yielded higher HA levels than the crude TW before and after egg passage. Infectivity levels of first amniotic fluids were generally low in the concentrates as well as in crude TW when compared to egg-adapted laboratory strains of influenza A_2. The relationship of these 2 parameters (I/HA) is also shown in Table I.

The results of a parallel study, to determine the sensitivity and reliability of the direct identification test, employing egg passage and the direct HA tests on 57 skim-milk TW and on 55 veal infusion TW are seen in Table II. There were 28 positives by egg passage and 26 by the direct HA test.

Discussion. The results of this study show that throat washings of individuals infected with influenza virus contain sufficient virus to produce a direct hemagglutination reaction. Usually the reaction is not obtained without concentration of the specimen. The data suggest that this procedure not only concentrates virus, but also separates it from one or more soluble, reversible inhibitors(3).

Titrations of infectivity and HA indicate that throat washings contain a higher number of HA units than egg infectious units. Since it requires about 10^6 viral particles per ml to agglutinate red cells, the throat washings must contain at least this number of viral particles. However, since the total num-

TABLE II. Comparison of Influenza A_2 Isolation Results Obtained from 57 Skim-Milk and from 55 Veal Infusion Broth Throat Washing Specimens, by Conventional Egg Passage and by Concentration Micro-Test.

Skim-milk throat washings*					Veal infusion throat washing				
No. of specimens	Egg passage		Concentration micro-test		No. of specimens	Egg passage		Concentration micro-test	
	Pos	Neg	Pos	Neg		Pos	Neg	Pos	Neg
57	16	41	14	43	55	12	43	12	43

* The concentration-micro-tests on skim-milk throat washings were done about 3 months after specimens were received and stored at −20°C.

ber of particles must be introduced into the egg in order to initiate infection, it means either that very few particles are infectious, or that the population as a whole is poorly adapted to the egg(4,5). It is of interest, therefore, that the progeny virus of the first egg passage also has a very low ratio of I/HA although somewhat higher than the original material. This suggests that the population is undergoing gradual adaptation to a new host manifested by the requirement for fewer viral particles to initiate infection of the egg (*i.e.*, an increasing I/HA ratio)(6).

A comparison of the direct and egg-passage tests (Table II) showed the direct method as sensitive and as reliable as the more time consuming egg-passage test. A similar direct technique has been applied to the identification of poliovirus with similar results(7).

Summary. A method of concentration and partial purification of influenza virus (A_2) found in throat washings is described. A microtechnique is presented for identification of virus contained in the concentrates.

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Production of Lactation by Non-Sedative Phenothiazine Derivatives.* (29815)

M. BEN-DAVID, S. DIKSTEIN AND F. G. SULMAN

Department of Applied Pharmacology, School of Pharmacy, Hebrew University, Jerusalem, Israel

According to recent observations, the hypothalamus contains a prolactin-inhibiting factor (P.I.F.)(1,2,3), which has been shown both *in vitro* and *in vivo*. *In vitro* the hypophysis-hypothalamus organ co-culture technique(1,2,4,5) has proved that presence of the hypothalamus inhibits prolactin secretion from the hypophysis. *In vivo*, however, mammary gland development and lactation can be achieved by: (a) hypothalamic lesions in rats and rabbits(6,7), (b) cutting of the pituitary stalk in women(8) and goats(9), (c) hypophyseal autografts in rats(10) and mice (11) at sites not controlled by the hypothala-

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