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Simplified Technique for Titrating Influenza Virus Neutralizing Antibody in the Chick Embryo.*

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Following the studies of Burnet[1] and Hirst,[2] the chick embryo has largely supplanted the mouse for estimating neutralizing antibodies for influenza virus. A method is described here which eliminates the necessity of harvesting fluid from each egg individually and testing it for virus.

The following strains of influenza virus were used in these tests and in preparation of rabbit sera: 2 classical influenza A strains (PR8 and Weiss), 2 strains of influenza A isolated during the outbreak of 1943 (MB and CP), 2 atypical strains of influenza A isolated early in 1947[3,4] (FM and EB) and the Lee strain of influenza B. All had been passed repeatedly by the allantoic route in eggs. Both human and rabbit immune sera were tested. Each of the latter was obtained 2 weeks after a single intravenous injection of 0.1 ml of virus infected allantoic fluid. Acute and convalescent sera were obtained from patients with clinical influenza in 1947.[4] The serum virus mixtures were prepared as described by Hirst.[2] The sera were heated at 60° C for 20 minutes; 4-fold dilutions were then made in saline; and a constant amount of virus (adjusted to contain 500-1000 allantoic 50% infecting doses in 0.1 cc of the final mixture) added to each of the serum dilutions and mixed thoroughly. The mixtures were then allowed to stand for a few minutes at room temperature; 0.1 ml was inoculated into the allantoic sac of 10-day-old embryos and 0.25 ml, or about 50-100 LD_{50}, was injected into the yolk sac of 7-day embryos. From 4 to 6 embryos were inoculated with each dilution. The virus suspension in 20% horse serum broth was titrated by inoculating 2 series of eggs in a similar manner with 10-fold serial dilutions, one series allantoically and the other by the yolk sac. Eggs were incubated at 35° C and embryos dying during the first 24 hours discarded.

At the end of 48 hours, allantoic fluids from eggs inoculated into the allantoic sac were tested for presence of virus by addition of ½ ml of 1% hen cells to ½ ml of the fluid. The 50% serum protective titer was then calculated by the method of Reed and Muench.[5] The yolk sac inoculated eggs were candled daily and the deaths recorded up to the fifth day after inoculation, when survivors were discarded. Tests of the yolk and/or allantoic fluids of dead eggs with hen cells revealed the presence of virus in the yolk in all instances and in the allantoic fluid in one-half of the eggs. Most of the survivors had virus demonstrable in the yolk but rarely in allantoic fluid. The 50% serum protective titer was then calculated.

Rabbit sera obtained before immunization and acute phase human sera showed titers of $<1:4$ in almost every instance. The comparative titers for neutralizing antibody obtained by the allantoic and yolk sac technics on the immune human and rabbit sera with different viruses are given in Table I.

It is of interest to point out first that the nonimmune rabbit sera and the acute

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1 Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1941, **19**, 39.

2 Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

3 Smadel, J. E., *Bull. U. S. Army Med. Dept.*, 1947, **7**, 795.

4 Morgan, H. R., Barnes, M. W., and Finland, M., *J. Lab. & Clin. Med.*, 1948, in press.

5 Reed, L. J., and Muench, A., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE I.
Comparison of Titers of Influenza Virus Neutralizing Antibodies in Human and Rabbit Immune Sera Using Allantoic and Yolk Sac Routes.

Rabbit serum	Neutralizing titer for influenza virus*					
	PR8†		Weiss†		MB†	
	Allantoic	Yolk sac	Allantoic	Yolk sac	Allantoic	Yolk sac
PR8	<4/2048	<4/>4096	<4/128	<4/810	—	—
Weiss	<4/8	<4/16	<4/128	<4/162	—	—
MB	<4/64	<4/64	<4/512	<4/1024	<4/2048	<4/>4096
CP	<4/512	<4/256	—	—	—	—
FM_1	<4/<4	<4/<4	—	—	<4/<4	<4/<4
EB	<4/<4	<4/<4	—	—	<4/4	<4/<4
Lee	<4/<4	<4/<4	—	—	—	—

Human serum	Neutralizing titer for influenza virus*					
	PR8†		EB†		FM_1†	
	Allantoic	Yolk sac	Allantoic	Yolk sac	Allantoic	Yolk sac
EB	<4/32	<4/91	<4/6	<4/28	11/181	4/64
GP	—	—	<4/<4	<4/23	—	—
DW	—	—	<4/<4	<4/<4	—	—
HB	<4/512	<4/512	<4/512	6/>1024	<4/160	<4/425
PW	<4/<4	<4/<4	<4/<4	<4/<4	—	—

* Expressed as reciprocals. Numerator is pre-immunization or acute phase titer; denominator is immune or convalescent titer. Each pair of sera tested simultaneously by both methods.

† For allantoic tests the dose was approximately 500 ID_{50} and for the yolk sac method about 50 LD_{50}.

—, not done.

phase human sera almost all failed to show neutralization by either the allantoic or yolk sac methods. This suggests that the presence of neutralizing antibody is a reliable index of recent antigenic exposure to influenza virus.

These data show that the presence of neutralizing antibodies for influenza viruses can be determined by the yolk sac technic and that this method has essentially the same range of sensitivity and specificity as the method employing the allantoic sac route of inoculation. The differences in the results obtained by the two methods is probably within the range of experimental error. Application of this method in a study of antigenic differences among strains obtained in an outbreak of influenza are presented elsewhere.[4]

Summary and conclusions. A yolk sac technic for the titration of serum neutralizing antibody for influenza viruses has been described which eliminates the necessity of testing each individual egg for infectivity since death of the embryo is used as a measure of infection with the virus.