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A Method for Increasing the Sensitivity of the Hemagglutination-Inhibition Test with Equine Influenza Virus[1]

Berlin *et al.* (*1*) and Werner *et al.* (*2*) described enhancement of hemagglutination-inhibiting titers by the use of ether-treated allantoic fluid suspensions of Asian influenza virus. Recently several strains of a new equine influenza virus[2] were found to be poorly inhibited by convalescent sera obtained from horses recovering from equine influenza. Therefore, the use of ether-treated allantoic fluid suspensions of equine virus was tried to determine whether serodiagnosis could be improved.

[1] This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported by the Office of the Surgeon General, U.S. Army, Washington 25, D. C.

[2] Isolated by James L. McQueen in Michigan during May, 1963.

Ether treatment was accomplished by forcibly mixing equal volumes of cold anesthetic ether (4°–10°C) and cold allantoic fluid suspension of virus in two precooled 20-cc Luer-Lok syringes. The syringes were coupled by a double-hubbed needle like that employed for preparing emulsified influenza virus vaccines (*3*). The syringes were laid horizontally on a table, and the contents of one syringe were pushed into the other. The liquids were forced through the double-hubbed needle six times with considerable pressure. The syringes then were placed in a refrigerator for approximately 5 minutes so that the contents had time to separate. Three layers were formed: the uppermost consisted of ether, the lowest contained the hemagglutinating activity, and the intermediate layer formed an opaque interphase. If the intermediate layer did not appear, the process of recycling the contents of the syringes was repeated using greater force. Recovery of treated allantoic fluid was accomplished by holding the syringes vertically and moving the aqueous phase into one syringe. That material was used directly without removing dissolved ether.

Hemagglutination (HA) titers and hemagglutination-inhibition (HAI) titers were measured by the standard pattern method using 0.5% chicken or pigeon erythrocytes (*4*). HA titers of treated virus suspensions were usually twofold to fourfold lower than those of the untreated suspensions, especially when measured with pigeon erythrocytes. However, in setting up the HAI tests, ether-treated and untreated virus suspensions were appropriately diluted so as to give preparations containing 16 units of hemagglutinating activity per milliliter with each type of cell.

Enhancement of HAI titers by the use of ether-treated material is illustrated in Table 1. In this experiment, HAI titers of acute and convalescent horse and ferret sera were measured simultaneously, treated and untreated virus suspensions and chicken erythrocytes being used. With the untreated virus suspension, sera from only the first five horses and from the ferrets showed a fourfold or greater rise in HAI activity. A positive serodiagnosis was made in those cases, but HAI levels from the remaining

TABLE 1

ENHANCEMENT OF HAI TITERS BY THE USE OF ETHER-TREATED EQUINE INFLUENZA VIRUS[a]

Animal number	HAI titer			
	Untreated virus		Ether-treated virus	
	Acute serum	Convalescent serum	Acute serum	Convalescent serum
Horse:				
1	<8	64	<8	128
2	<8	32	<8	256
3	<8	32	<8	128
4	<8	16	<8	128
5	<8	16	<8	64
6	<8	8	<8	32
7	<8	8	<8	32
8	<8	8	<8	24
9	<8	<8	<8	32
Ferret:				
1	<8	32	<8	128
2	<8	16	<8	128
3	<8	16	<8	64

[a] A/Eq/Det. 2/63.

TABLE 2

COMPARISON OF HAI TITERS OBTAINED WITH CHICKEN AND PIGEON ERYTHROCYTES

Viral antigen[a]	HAI titer			
	Chicken cell		Pigeon cell	
	Horse serum[b]	Ferret serum[b]	Horse serum[b]	Ferret serum[b]
Untreated	60	20	160	60
Treated	480	320	1280	320

[a] A/Eq/Det. 2/63.

[b] Convalescent sera.

four horses were too low to permit a decision; in one case, antibody was not detected. By comparison, HAI titers of individual convalescent sera with ether-treated material ranged from twofold to sixteenfold higher than those obtained with untreated virus. Moreover, the antigen did not become sensitive to nonspecific inhibitors after treatment with ether.

Evidence that enhancement of HAI titers was due to a direct effect on viral antigen rather than indirectly through some nonviral component of allantoic fluid rests on several observations. Virus that had been concentrated and purified by high speed centrifugation and then treated with ether was as effective an antigen as uncentrifuged material similarly treated with ether. On the other hand, concentrated virus that was mixed with ether-treated allantoic fluid from which virus had been removed by centrifugation was no more effective than untreated uncentrifuged material. Furthermore, diluting the antiserum in 0.01 *M* phosphate-buffered saline saturated with ether did not enhance HAI titers.

An additional fourfold increase in sensitivity of the HAI test was obtained by combining the use of pigeon erythrocytes and ether-treated influenza virus. The use of pigeon red cells reportedly enhances HAI titers obtained with untreated influenza virus suspensions (*5*). In this laboratory HAI titers of convalescent horse serum and ferret serum tested with pigeon red cells were approximately twofold higher than those obtained using chicken red cells (Table 2). Furthermore HAI titers obtained with pigeon erythrocytes and ether-treated virus were at least sixteenfold higher than those observed with chicken erythrocytes and untreated virus. The additive nature of these phenomena is intriguing and suggests avenues for further investigations.

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Accepted October 1, 1963