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IMPROVEMENT OF THE METHOD OF PLAQUE FORMATION OF INFLUENZA VIRUS AND ITS APPLICATION TO NEUTRALIZATION TESTS

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SUMMARY The plaque method for influenza virus using pancreatin and agarose in culture medium for chick embryo cells (Came et al., 1968) was improved. Addition of skim milk to Medium 199 enhanced the plating efficiency of many influenza strains. The plating efficiencies in LLCMK2 cells and quail embryo fibroblast cells were comparable to that in chick embryo cells.

Various conditions of the plaque method in chick embryo cell cultures were investigated and the method was applied to a neutralization test. The neutralizing antibody titers of human sera by the plaque method were generally parallel with their hemagglutination inhibition titers for various strains. The potency of killed influenza vaccine could be measured reliably and conveniently by the plaque neutralization method using sera of mice inoculated with serial dilutions of the vaccine.

INTRODUCTION

Plaque formation of influenza virus has been studied by many investigators, but most studies were limited to a few strains of the virus, to continuous cell lines, or of low PFU/EID_{50} ratios (Simpson and Hirst, 1961, Choppin, 1962, Lehmann-Grube, 1963, Takemoto and Fabisch, 1963, Vonka, 1964, Grossberg, 1964, Sugiura and Kilbourne, 1965, Hatano and Morita, 1967). Came et al. (1968) recently reported that pancreatin and agarose in overlay medium enhanced the number and size of plaques in chick embryo cell cultures. In the present study their method was improved to give higher PFU/EID_{50} ratios, and was applied to the neutralization (NT) test of human sera. The NT titers obtained by the plaque method were compared with the hemagglutination inhibition (HI) titers, and the plaque neut-

ralization method was applied to the potency test of killed influenza vaccine.

MATERIALS AND METHODS

1. *Preparation of chick embryo fibroblast (CEF) and quail embryo fibroblast (QEF) cultures*

Decapitated 10 days-old chick embryos were minced with scissors and digested with trypsin at 37 C. The dispersed cells were centrifuged at 1,500 rev/min for 10 minutes and resuspended in growth medium. The suspensions were filtered through a 200 mesh-filter and diluted with growth medium to a concentration of $3–4 \times 10^6$ cells per ml. Two-ounce rubber stoppered bottles or 60 mm plastic dishes (Toyoshima Works, Tokyo, Japan) were seeded with 7 ml and 5 ml of the cell suspension, respectively. The bottle cultures were incubated at 37 C, and the dish cultures were placed in a humidified atmosphere of 5% CO_2. QEF cultures were prepared by the same procedure.

2. *Preparation of LLCMK2 cells*

LLCMK2 cells (a cell line from rhesus monkey kidney) were kindly supplied by Dr. S. Nii, who originally obtained them from Dr. P. K. Russel. Cell were grown in Medium 199 (M-199) (Difco Lab.) containing 10 per cent calf serum, and 7 ml of cell suspersion containing 1.5×10^5 cells per ml were seeded into two-ounce bottles with rubber stoppers. The bottles were incubated at 37 C and virus was inoculated on the 3rd day.

3. *Viruses*

Various strains of influenza virus of types A, A1, A2 and B used in this study were originally supplied from the National Institute of Health of Japan, and serially passaged in chick embryo allantoic cavity in our laboratory.

4. *Culture media and diluent*

Chick embryo fibroblast (CEF) cultures and quail embryo fibroblast (QEF) cultures were grown in Eagle's MEM, containing 0.3% tryptose phosphate broth (Difco Lab.) and 5% calf serum, and LLC-MK_2 cells were grown in M-199 containing 10% calf serum. Virus samples and serum were diluted with phosphate buffered saline containing 0.02% gelatine.

5. *Pancreatin*

Pancreatin was obtained as a powder from Nutritional Biochemicals and Difco. Five percent pancreatin solution was prepared in phosphate buffered saline. The pancreatin was allowed to dissolve overnight at 4 C, filtered through a 0.45 μ Millipore filter, and frozen as a stock solution.

6. *Plaque assay*

Growth medium was removed and cultures were washed with phosphate buffered saline, and inoculated with 0.2 ml of serially diluted virus suspension or virus-serum mixture. After adsorption at 37 C for 60 to 120 min., 5 ml overlay medium was added. The overlay medium consisted of a mixture of equal volumes of 1.8% agarose and double concentration M-199 without phenol red, containing 0.3% $NaHCO_3$, 4% skim milk (Difco Lab.), 0.006% neutral red, 200 units penicillin and 200 μg kanamycin. After addition of the overlay medium, the cultures were incubated at 37 C for 3 to 5 days, then the number of plaques was counted.

RESULTS

1. *Effects of various concentrations of pancreatin*

The effects of various concentrations of pancreatin on plaque formation are shown in Table 1. Pancreatin activity varied somewhat in different batches and its available concentration was limited. Generally a concentration of 0.03% seemed most suitable for plaque formation in chick cell cultures.

When skim milk was omitted from the overlay medium, plaques were more diffuse and the plating efficiency was usually low. Noble agar (Difco), Bactoagar (Difco) and agarose were compared as solidifying agents. With agarose plaque appeared most rapidly and plaque size was the largest.

2. *Comparison of the bottle method with the dish method*

Two-ounce bottle celtures and 60 mm plastic dish cultures were inoculated simultaneously with the same dilutions of virus, and after addition of the overlay medium, the cultures were incubated at 37 C. Dish cultures

TABLE 1. *Effects of various concentratins of pancreatin on plaque formation*

Virus strain	Pancreatin product (Lot)	Concentration of pancreatin in overlay medium (%)				
		0.01	0.02	0.03	0.04	0.05
B/Tokyo/1/67	A	62[a] (2.5)[b]	98 (3.0)	110 (2.5)	85 (1.5)	69 (0.5)
	B	30 (1.5)	109 (3.0)	112 (2.0)	126 (1.5)	107 (1.0)
	C	4 (2.4)	123 (3.5)	119 (3.0)	117 (2.0)	109 (1.5)
A2/Aichi/2/68	A	98 (2.0)	92 (2.5)	85 (1.5)	82 (1.0)	78 (1.0)
	B	77 (2.0)	73 (2.0)	83 (1.5)	63 (1.0)	42 (1.0)
	C	2 (2.0)	83 (2.0)	98 (2.5)	96 (2.5)	90 (3.0)

a: Average plaque number in 2 bottles
b: Average plaque diameter (mm)

TABLE 2. *Comparison of the plating efficiencies of influenza viruses using the bottle method and the dish method*

Virus strain	PFU/ml		(a)/(b)
	Bottle method (a)	Dish method (b)	
A/RR8/34	2.0×10^5	1.5×10^5	1.33
A1/Ohmachi/1/53	4.2×10^5	2.9×10^5	1.45
A2/Adachi/1/57	4.4×10^5	3.5×10^5	1.26
A2/Murakami/4/64	2.8×10^5	1.5×10^5	1.87
A2/Aichi/2/68	1.3×10^6	4.4×10^5	2.95

were placed in a humidified atmosphere under 5% CO_2. After incubation, the numbers of plaques in the two types of cultures were compared. As shown in Table 2, the plating efficiency was generally a little higher by the bottle method than by the dish method. Moreover, plaques appeared more rapidly and were larger in bottle cultures. The bottle method was also usually more convenient for the treatment of a large quantities of samples. Therefore, all subsequent neutralizations using the plaque method were done with the bottle cultures.

3. *Efficiency of plaque formation relative to the egg infectious dose*

When virus stocks were prepared by passage through the chorioallantoic cavity, the ratio of plaque forming units (PFU) to the 50% egg infectious dose (EID_{50}) was over 0.1 for many strains. However, for some strains, such as A1/Ohmachi/1/53 and A2/Murakami/4/64, the ratio was lower and the plaques were diffuse and not uniform in size. With these strains, when virus stocks were prepared from virus suspensions obtained by mixing a few selected clear plaques, the ratio was greatly enhanced (Table 3). Several clones of virus were mixed to avoid the possible deviation of antigenicity when only a single clone was taken for preparation of virus stocks. No difference was observed between the antigenicities of the original virus stocks and those of mixed clones by the cross HI test.

4. *Plaque formation of influenza viruses in LLCMK2 cells and quail embryo fibroblast cells*

Plaque formation of influenza viruses was tested in LLCMK2 cells and quail embryo fibroblast cells. Virus stocks were prepared from viruses which had been serially passaged

TABLE 3. *Comparison of PFU in CEF cultures with EID_{50} of various influenza strains*

Virus strain	Virus stock (a)			Virus stock (b)		
	PFU/ml	EID_{50}/ml	PFU/EID_{50}	PFU/ml	EID_{50}/ml	PFU/EID_{50}
A/PR 8/34	1.2×10^9	2.3×10^8	5.2	2.5×10^8	2.1×10^9	0.12
A1/Ohmachi/1/53	2.1×10^7	3.4×10^9	0.0062	3.2×10^8	8.9×10^8	0.36
A2/Adachi/2/57	1.8×10^8	1.5×10^{10}	0.012	2.4×10^8	2.5×10^9	0.096
A2/Murakami/4/64	2.5×10^6	1.5×10^{10}	0.0002	1.7×10^8	8.5×10^8	0.20
A2/Kumamoto/1/65	9.6×10^7	3.4×10^9	0.028	9.3×10^7	1.7×10^9	0.055
A2/Kumamoto/1/67	1.9×10^8	1.6×10^9	0.12	2.6×10^8	3.8×10^9	0.068
A2/Aichi/2/68	2.9×10^8	2.1×10^9	0.14	1.1×10^9	5.1×10^9	0.22
B/Sapporo/65	8.1×10^6	1.6×10^7	0.51	1.4×10^8	1.6×10^8	0.88
B/Tokyo/1/67	5.5×10^8	1.2×10^9	0.46	1.2×10^8	1.9×10^9	0.063

Virus stock
(a): Serially passed through chorioallantoic cavity.
(b): Passed once through plaque in CEF cultures from virus stock (a) and cultured in chorioallantoic cavity.

TABLE 4. *Comparison of PFU in LLCMK2, in CEF, in monkey kidney (MK) cultures and EID_{50}*

Virus strain	Cultures (PFU/ml)			EID_{50}/ml
	LLCMK2	CEF	MK	
A2/Kumamoto/1/67	4.5×10^5	5.5×10^6	NT[a]	1.6×10^8
A2/Aichi/2/68	2.4×10^8	2.6×10^7	NT	3.4×10^8
A1/Ohmachi/1/53	5.0×10^6	2.4×10^5	NT	1.6×10^8
B/Amakusa/1/64	1.4×10^6	7.1×10^6	2.1×10^4	7.4×10^6

a NT: Not tested.

TABLE 5. *Comparison of PFU in chick embryo fibroblast cells (CEF) and quail embryo fibroblast cells (QEF)*

Virus strain	PFU/ml		CEF/QEF
	CEF	QEF	
A2/Adachi/1/57	4.0×10^5	4.0×10^5	1.0
A2/Kumamoto/1/67	1.8×10^5	2.7×10^5	0.67
A2/Aichi/2/68	3.9×10^5	4.6×10^5	0.85
B/Sapporo/1/65	2.3×10^5	2.1×10^5	1.10
B/Tokyo/1/67	1.4×10^6	1.7×10^5	0.82

through the chorioallantoic cavity. As shown in Tables 4 and 5, the plaque efficiency in these two cells were generally comparable to that in CEF cells. However, CEF cells were readily available in large quantities, and so all subsequent neutralization tests were performed with CEF cells.

5. *Neutralization test*

Details of the procedure used in the neutralization test are shown in Fig. 1. Briefly, sera were inactivated by heating at 56 C for 30 minutes, and diluted serially 2 or 4 fold. Then an equal volume of challenge virus was added to the serum, and the mixture was incubated at room temperature for 60 minutes and at 4 C overnight. Two bottles of CEF culture were used for each serum sample and were inoculated with 0.2 ml of mixture per bottle. The serum dilution which reduced the plaque number by 50% was taken as the neut-

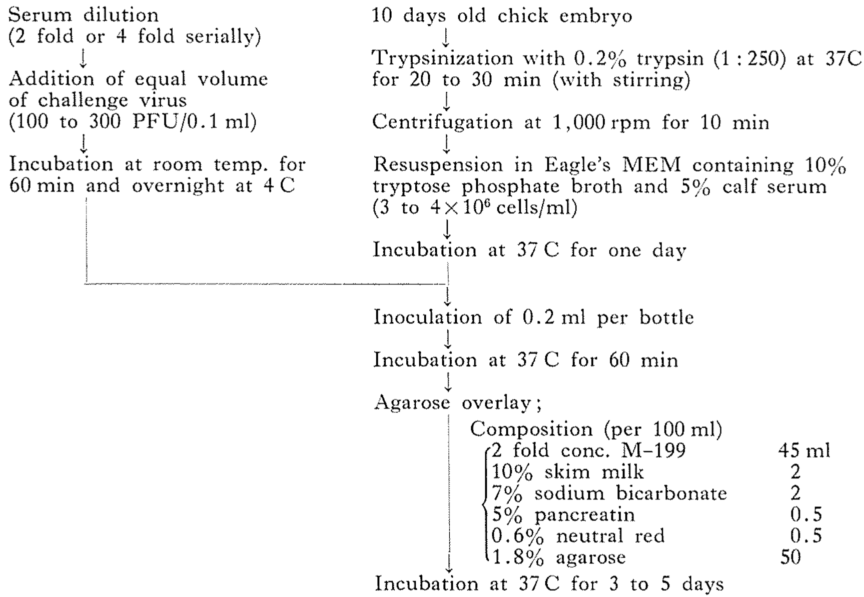


FIGURE 1. *Schedule for neutralization test of influenza viruses by the plaque method*

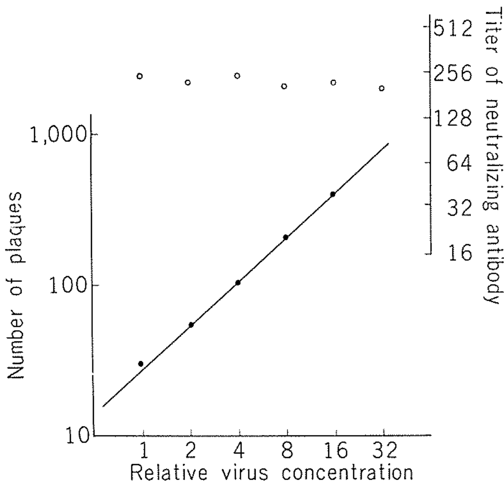


FIGURE 2. *Relation of the concentration of influenza virus A2/Aichi/2/68 strain to the number of plaques in CEF cultures, and to the neutralizing antibody titer of human serum inoculated with a killed influenza virus vaccine.*

ralizing antibody titer. The relationship between the dose of challenge virus and the neutralizing antibody titer is given in Fig. 2. When the dose of challenge virus was between 30 to 800 PFU, the antibody titer was usually within 2 fold dilution of serum. From this result, a dose of challenge virus of 100 to 300 PFU/0.1 ml was used in neutralization tests.

6. *Comparison of the neutralizing (NT) antibody titer by the plaque method with the hemagglutination inhibition (HI) antibody titer*

Sera from patients with Hong Kong type influenza virus (acute and convalescent), and from subjects vaccinated with killed influenza vaccine (containing 200 C.C.A. units of A2/Aichi/2/68, 50 C.C.A. units of B/Tokyo/7/66 and 50 C.C.A. units of B/Tokyo/1/67 per ml) were tested for NT and HI antibody titers. As shown in Figs. 3 and 4, the NT antibody titer was generally proportional to the HI antibody titer in these sera. Furthermore an ap-

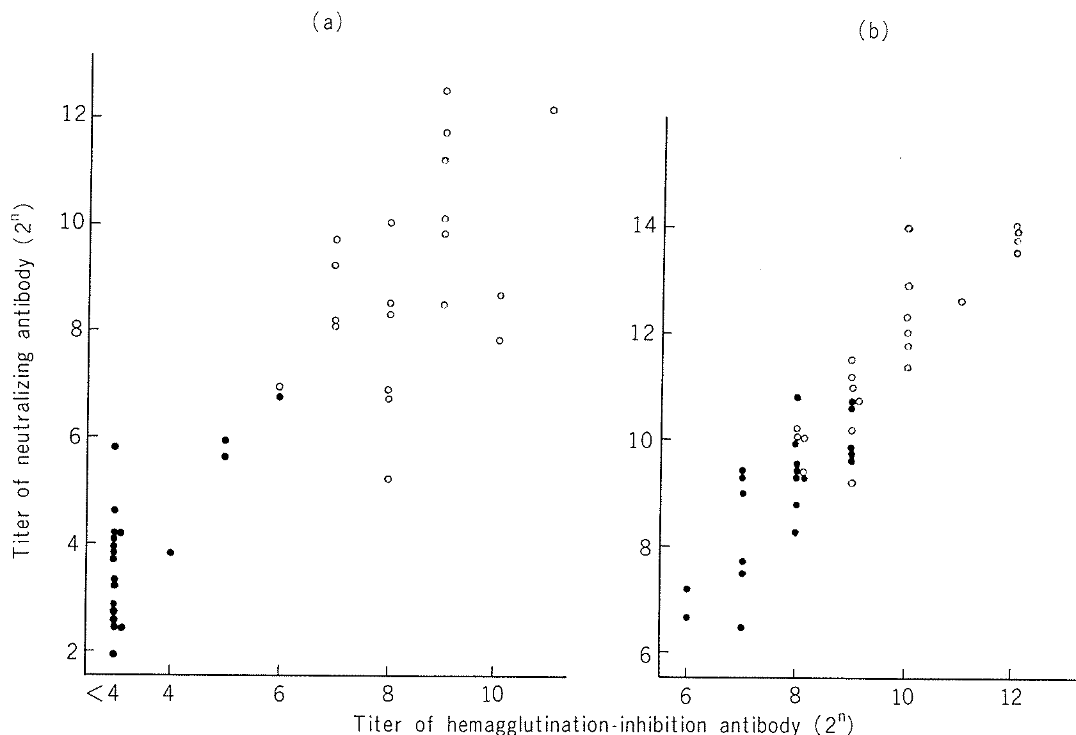


FIGURE 3. *Comparison of neutralizing antibody titer with hemagglutination-inhibition antibody titer of sera of patients with Hong Kong type influenza.*
The patients had Hong Kong type influenza from December 1968 through February 1969.
(●) *Sera at acute stage* (○) *Sera at convalescent stage*
Challenge virus; (a) A2/Aichi/2/68 (Hong Kong type variant) (b) A2/Kumamoto/1/67

parent difference between the antigenicities of A2/Aichi/2/68 (Hong Kong type variant) and A2/Kumamoto/1/67 by the NT test and HI test was noted.

7. *Application of the plaque neutralization method to test the potency of killed influenza vaccine*

The possibility of application of the plaque neutralization method to test the potency of killed influenza vaccine was studied. Killed influenza vaccine (containing 200 C.C.A. units of A2/Aichi/2/68, 50 C.C.A. units of B/Tokyo/7/66 and 50 C.C.A. units of B/Tokyo/1/67 per ml) was serially diluted 5 fold. Aliquots of 0.5 ml of each dilution were injected intraperitoneally into 10 mice, which were bled 14 days after injection. The serum was then subjected to the plaque neutralization test with the A2/Aichi/2/68 strain.

As shown in Fig. 5, the neutralizing antibody titers by the plaque reduction method were proportional to the dilution of injected vaccine, indicating that neutralization by the plaque reduction method can be used to test the potency of killed vaccine.

▶

FIGURE 5. *Antibody response of mice to serial dilutions of killed vaccine of influenza virus A2/Aichi/2/68. Mice were inoculated with serial 5 fold dilutions of vaccine and bled 2 weeks later.*
(●) *Antibody titer of individual sera*
(○) *Geometric mean of antibody titer*
(×) *Antibody titer of pooled sera*

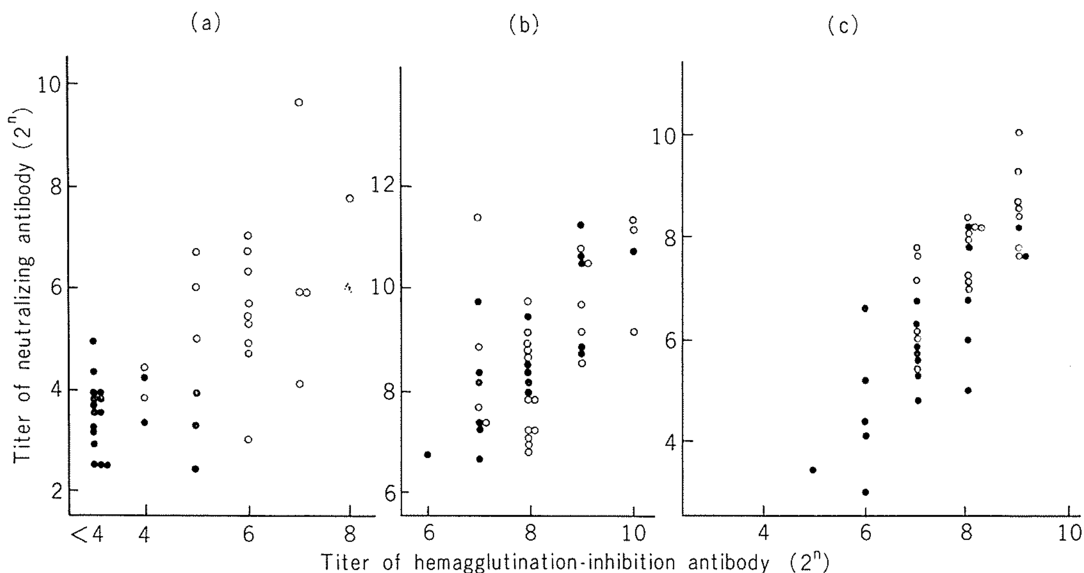
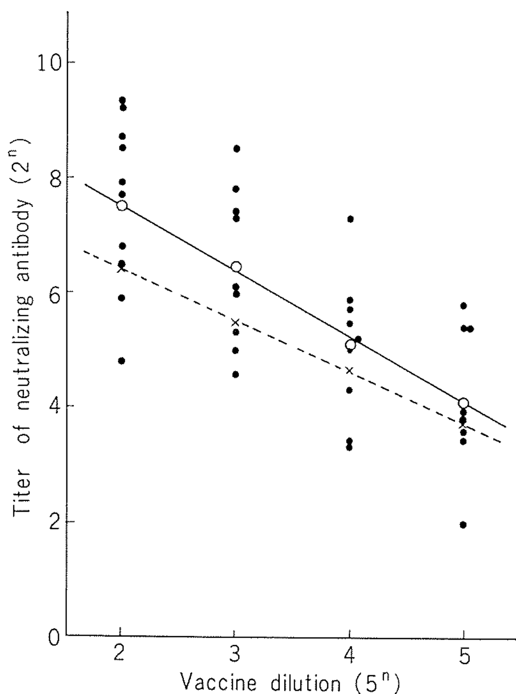


FIGURE 4. *Comparison of neutralizing antibody titers with hemagglutination inhibition antibody titers of sera of subjects vaccinated with killed influenza vaccine.*
The vaccine contained A2/Aichi/2/68, B/Tokyo/7/66 and B/Tokyo/1/67 strains.
(●) *Sera before vaccination,* (○) *Sera after vaccination.* *Challenge viruses;* (*a*) *A2/Aichi/2/68* (*b*) *A2/Kumamoto/1/67* (*c*) *B/Tokyo/1/67*



DISCUSSION

Came et al. (1968) reported that incorporation of pancreatin into the overlay medium of chick embryo monolayers enhanced the number and size of plaques of influenza virus. In the present study their method was improved to give a higher PFU/EID_{50} ratio for many influenza virus strains. Using M-199 medium and skim milk in addition to pancreatin and agarose, PFU/EID_{50} ratios of over 0.1 were obtained with many influenza strains. These values of the PFU/EID_{50} ratio suggest that 1 PFU is nearly equal to 1 EIU (Egg Infectious Units), since titers expressed as EID_{50} are 1.4 higher than those expressed as PFU, and after a two hour incubation period, virus adsorption is not yet complete. However, with some strains, such as A1/Ohmachi/1/53 and A2/Murakami/4/64, the ratio was still less than 0.01. When virus stocks were prepared from virus suspensions obtained by mixing a few selected clear

plaques on chick embryo cultures, the ratios were greatly enhanced. This indicates that the populations of the original virus stocks are not homogeneous and that the virus population efficient for plaque formation can be obtained by cloning.

Based on these results, accurate neutralization test became possible using plaque method. Neutralization tests on human sera were carried out using strains A2/Aichi/2/68, A2/Kumamoto/1/67 and B2/Tokyo/1/67, which are currently used as killed vaccine. The results indicated that the neutralizing antibody titers were roughly comparable with the hemagglutination inhibition titer. The potency of killed vaccine could also be measured by the plaque neutralization method. A linear relationship was found between the dilution of vaccine and the NT antibody titer. In practice, NT tests by the plaque method seem less laborious and more accurate than those using eggs.

Chick embryo cells are readily available in large quantities. This plaque method using chick embryo cells may be used in genetical studies on influenza virus, and in quantitative assays of infectious virus and the antibody titer, especially in secretions in which large amounts of nonspecific hemagglutination inhibitor might be present.

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