

CHAPTER IV

RESULTS

Characteristics of duck plaque virus in cell cultures

When a liver suspension containing duck plague virus was inoculated into DEF, a typical CPE began 2 or 3 days after infection, with foci of rounded, clumped cells. Careful examination revealed that syncytial formations and intranuclear inclusion bodies could be found. Given a time lapse of more than 72 hours, the area of necrosis and cellular debris became obvious.

Also, the duck plague attenuated virus produced CPE on CEF in the same way as the homologous virus on DEF.

Characteristics of duck plague virus infection as determined by post mortem findings

Death usually began on the 4th day after a challenge virus inoculation. The most common post mortem lesions were annular bands of hemorrhage encircling the intestinal lumen. Pronounced diphtheritic changes in the mucosae of the esophagus and cloaca were also present. Hemorrhages in the internal organs were present in some cases.

Inactivation of duck plaque virus

Virulent duck plague virus in DEF was inactivated by BEI at 37 °C after 4 hours (Figure 1) since no infectivity in DEF could be detected after 4 hours of inactivation. Virus control fluctuated

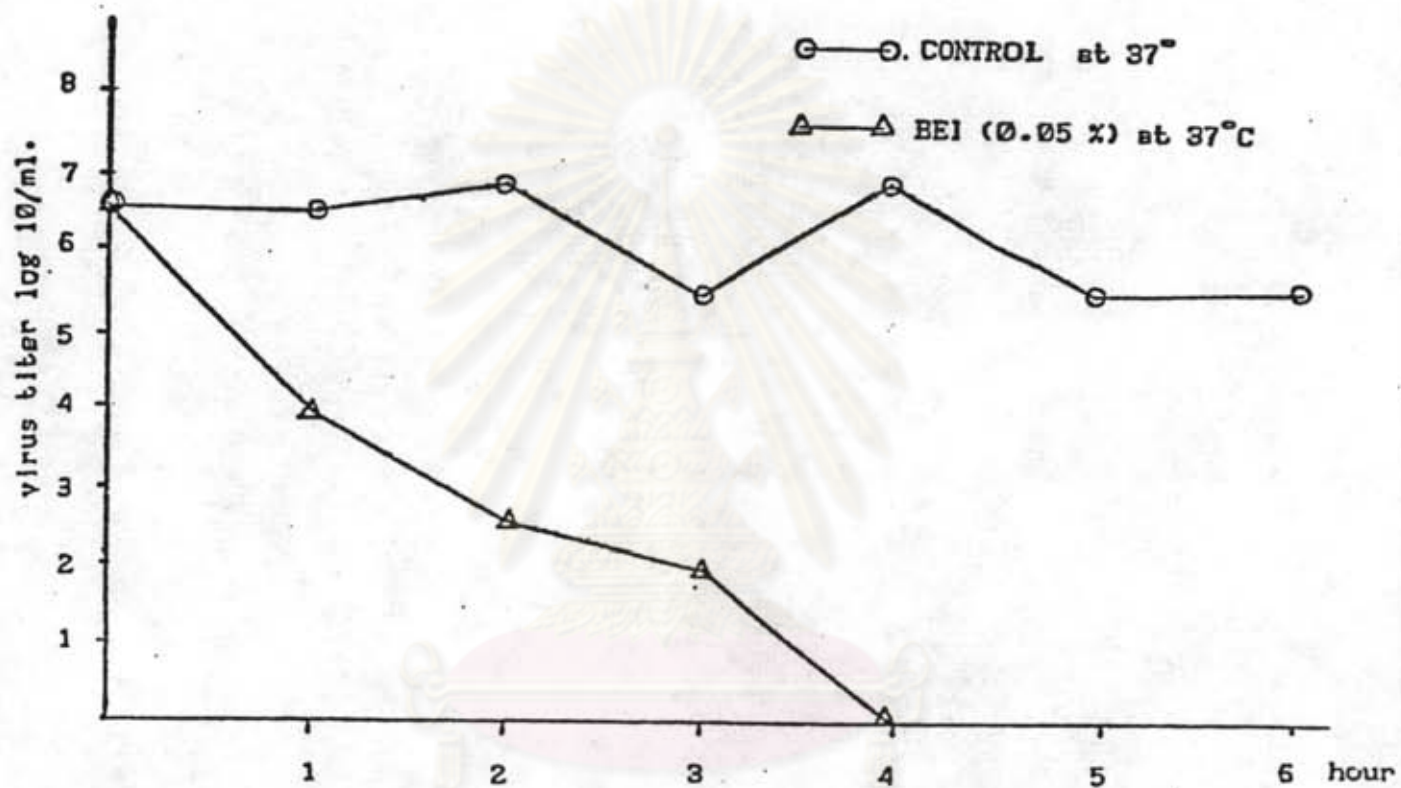


Figure 1 Inactivation of duck plague virus with 0.05 % BEI at 37° C for 6 hours

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slightly but its titer was approximately between $10^{5.6}$ and $10^{7.2}$ TCID₅₀ /ml. In the preparation of the vaccine, 6 hours BEI-inactivated vaccine was used for immunization.

Determination of titer of various vaccines

For attenuated vaccine, DP-L, the titer, after being reconstituted with 1 ml of PBS, was $10^{4.5}$ TCID₅₀. So 1 dose (1 ml) of vaccine contained $10^{2.2}$ TCID₅₀.

The titer of virulent duck plague virus in DEF before inactivation was $10^{7.0}$ TCID₅₀ /ml or $10^{5.0}$ DLD₅₀ /ml so 0.5 ml injection of inactivated vaccine without oil, BEI-DP, contained $10^{6.7}$ TCID₅₀ based on inactivation control titer. Due to the fact that this virus was also used to prepare the oil vaccine, BEI-DPM, the virus content in 1 ml of immunization was equivalent to BEI-DP.

Neutralization index and the rate of survival of various vaccinated groups

Two months after the first vaccination, NI of various vaccinated groups were not significantly different from the control group except for that of the BEI-DPM group. However, the protective immunity of the DP-L group was higher than both the BEI-DP and BEI-DPM groups. The attenuated vaccine, DP-L, gave 85-100% protection for upto 2 months, whereas the inactivated vaccine without oil, BEI-DP, and the inactivated vaccine with oil, BEI-DPM, gave 42-71% and 42-75% protection for up to 2 months, respectively, NI and the rate of survival after the first vaccination within the first 2 months and the relationship between the antibody titer (determined by neutralization) and the immunity are shown in Tables 1,2 and Figure 2.

Table 1 Neutralization index ($\bar{X} \pm SD$) of duck serums after the first vaccination

Vaccine	before vaccination	NI (No. serum tested)		
		after 1 st vaccination		
		2 weeks	1 month	2 months
DP-L	} 0.66±0.29(124)	0.83±0.43(29)	0.41±0.33(24)	0.22±0.21(34)
BEI-DP		0.97±0.62(30)	0.63±0.52(35)	0.30±0.26(30)
BEI-DPM		2.06±1.07(26)	2.05±1.02(31)	1.13±0.76(31)
CONTROL		0.55±0.23(26)	0.30±0.26(34)	0.13±0.16(31)

Table 2 Protective immunity of ducks to duck plague virus after the first vaccination

Vaccine	No. survived/No. challenge inoculated		
	(% survival)		
	2 weeks	1 month	2 months
DP-L	8/8 (100)	7/8 (87.5)	6/7 (85.7)
BEI-DP	4/8 (50)	3/7 (42.8)	5/7 (71.4)
BEI-DPM	6/8 (75)	6/8 (75)	3/7 (42.8)
CONTROL	0/8 (0)	0/9 (0)	0/4 (0)

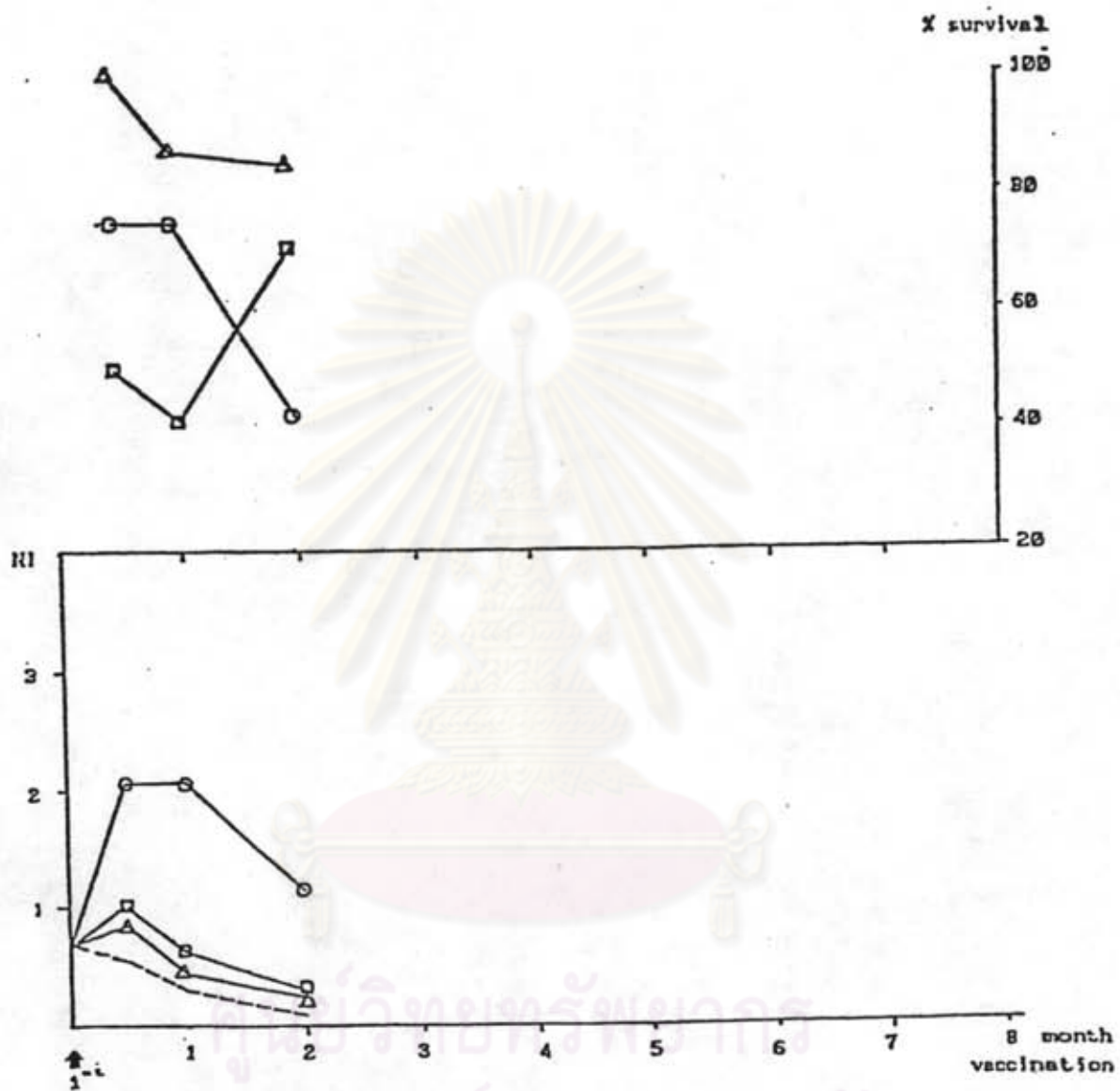


Figure 2 Comparison between NI and % survival of groups of various, vaccinated ducks from the first vaccination throughout the postvaccination period

100% mortality in control was observed

△—△ DP-L, □—□ BEI-DP, ○—○ BEI-DPM,

---- CONTROL

After the second vaccination, the outbreak occurred. The loss of ducks vaccinated once with DP-L, BEI-DP and BEI-DPM vaccine was 50% (4/8), 71% (5/7) and 43% (3/7), respectively. Of the surviving ducks, their NI were all higher than 3.0 regardless of the type of vaccine used. The increase in titer was certainly due to infection. However, the immunity of those ducks that survived from infection was not always solid immune. (Table 3 and Figure 3)



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Table 3 NI and % survival of groups of various vaccinated ducks at the 3rd month after the first vaccination (1 month after the outbreak occurred)

Vaccine	NI (No. serum tested)	No. survived/No. challenge inoculated (% survival)
DP-L	3.98±0.55 (4)	4/4 (100)
BEI-DP	3.80±1.41 (2)	2/2 (100)
BEI-DPM	3.55±0.40 (4)	2/4 (50)
CONTACTED CONTROL	2.40±1.69 (2)	Not tested
SEPERATED CONTROL	0.37±0.26 (26)	0/5 (0)

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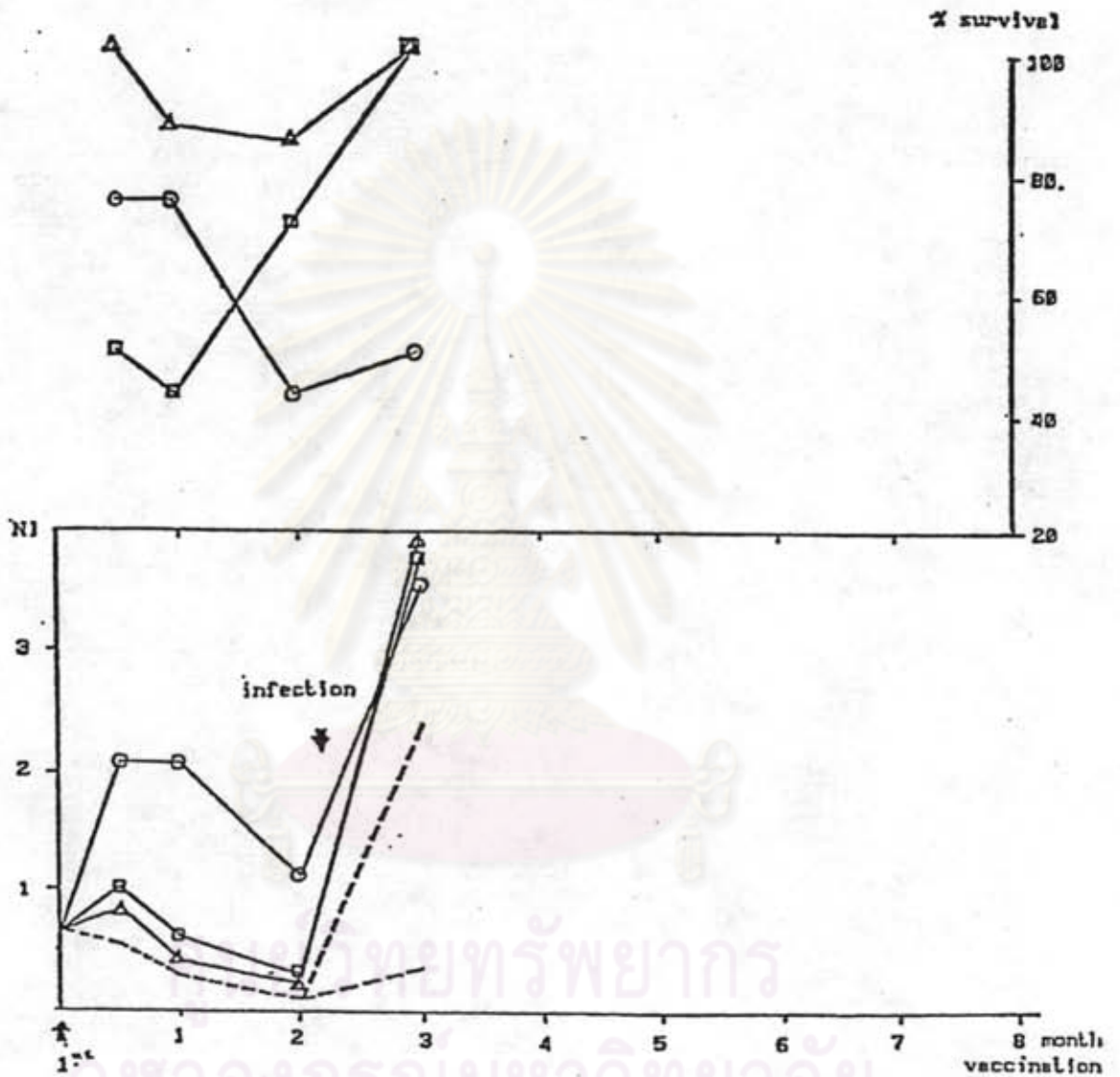


Figure 3 Comparison between NI and % survival of groups of various vaccinated ducks from the first vaccination throughout the postvaccination period (including the outbreak)

100% mortality in separated control was observed

△—△ DP-L, □—□ BEI-DP, ○—○ BEI-DPM,

----- SEPERATED CONTROL, -.-.- CONTACTED CONTROL

The mortality among the ducks that had been vaccinated twice (because of incomplete inactivation of virus) was low in the DP-L and BEI-DPM groups (4/66, 6% and 4/67, 6% respectively) but high in the BEI-DP group (33/66, 50%). The NI of every duck in all groups increased to a high level; approaching its the highest peak within 1 month. Six months later, although the average NI per group decreased slightly, their titer persisted between 1.5-3.0. Moreover, the difference between the NI among 3 groups of vaccine including the contacted control (2 ducks) that survived from the outbreak was not statistically significant ($p>0.05$) (Table 4)



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Table 4 Neutralization index ($\bar{X} \pm SD$) of duck serums after second vaccination

Vaccine	before 2 nd vaccination	NI (No. serum tested)						
		1 month	2 months	3 months	3 1/2 months	4 months	5 months	6 months
DP-L	0.22±0.21(34)	2.84±0.86(29)	1.29±0.75(34)	1.74±0.78(23)	2.10±1.07(7)	2.13±0.88(11)	1.79±0.91(10)	2.43±1.10(3)
BEI-DP	0.30±0.26(30)	3.25±0.77(23)	1.98±0.78(24)	2.07±0.83(20)	2.48±0.92(4)	2.78±0.59(8)	2.38±1.02(8)	2.77±0.73(4)
BEI-DPH	1.13±0.76(31)	3.12±0.64(27)	1.35±0.75(34)	1.56±0.69(22)	1.38±1.0(9)	1.75±0.92(11)	1.57±1.14(11)	1.26±0.94(3)
SEPERATED CONTROL	-	0.37±0.26(26)	0.14±0.23(18)	0.12±0.23(21)	0.04±0.08(9)	0.16±0.24(13)	0.19±0.18(8)	0.13±0.35(8)
CONTACTED CONTROL	0.13±0.16(31)	2.40±1.69(2)	1.76±1.16(2)	2.0±0.49(2)	2.0±0.42(2)	2.5±0.8(2)	1.8±0.5(2)	1.8±0.6(2)

The degree of resistance against duck plague challenge virus following the second vaccination varied. The protective immunity of the DP-L group was 75-100% for up to 6 months, whereas in the BEI-DP and BEI-DPM groups, it was 50-100% and 50-87%, respectively, over the same period. Obviously, the attenuated vaccine afforded a little higher level of protection than both types of inactivated vaccine (Table 5 and Figure 4).



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Table 5 Protective immunity of ducks to duck plague virus after the second vaccination

vaccine	No. survived/No. challenge inoculated (% survival)					
	1 month	2 months	3 months	4 months	5 months	6 months
DP-L	8/8 (100)	5/6 (83.4)	4/5 (80)	5/8 (100)	4/5 (80)	3/4 (75)
BEI-DP	6/6 (100)	3/6 (50)	Not tested	4/4 (100)	Not tested	4/5 (80)
BEI-DPM	5/7 (71.4)	7/8 (87.5)	5/6 (83.4)	3/5 (60)	6/7 (85.7)	3/6 (50)
SEPERATED	0/5 (0)	0/6 (0)	0/6 (0)	0/8 (0)	0/8 (0)	0/8 (0)
CONTROL						

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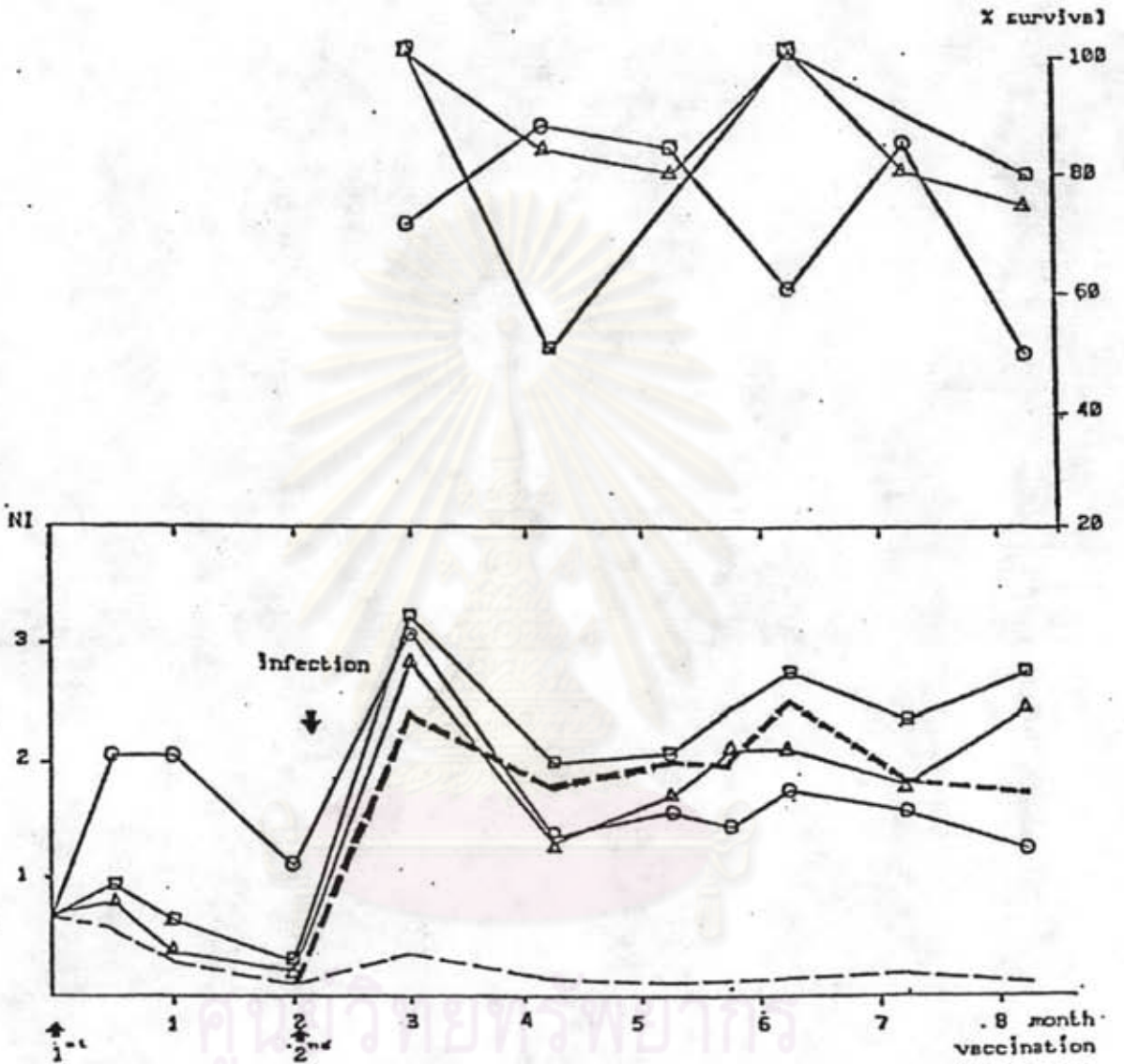


Figure 4 Comparison between NI and % survival of groups of various, vaccinated ducks from the second vaccination throughout the postvaccination period

100% mortality in seperated control was observed

△—△ DP-L, □—□ BEI-DP, ○—○ BEI-DPM,

----- SEPERATED CONTROL, -.-.- CONTACTED CONTROL

For the third vaccination, the average NI of both the BEI-DP and BEI-DPM groups was higher than DP-L group significantly ($p < 0.5$) over a period of 3 months. The immunity of all 3 groups of vaccine was quite low; with DP-L, the level of protection was 33-66% over a period of 3 months, whereas with BEI-DP and BEI-DPM, it was 40-100% and 33-100% respectively, over the same period. (Tables 6, 7, Figure 5).



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Table 6 Neutralization index ($\bar{X} \pm SD$) of duck serums after the third vaccination

Vaccine	NI (No. serum tested)				
	rd before 3 vaccination	rd 1/2 month	rd after 3 1 month	vaccination 2 months 3 months	
DP-L	1.74±0.78(23)	2.37±0.39(23)	2.02±0.55(13)	1.75±0.65(10)	1.8±0.79(6)
BEI-DP	2.07±0.83(20)	3.23±0.64(9)	2.66±0.92(10)	2.83±0.44(9)	2.12±0.45(5)
BEI-DPM	1.56±0.69(22)	2.57±1.01(7)	3.11±1.42(9)	2.78±1.15(9)	2.6±0.36(3)
SEPERATED	0.12±0.23(21)	0.04±0.08(9)	0.16±0.24(13)	0.19±0.18(8)	0.13±0.35(8)
CONTROL					
CONTACTED	2.0±0.49(2)	2.0±0.42(2)	2.5±0.8(2)	1.8±0.5(2)	1.8±0.6(2)
CONTROL					

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Table 7 Protective immunity of ducks to duck plague virus after the third vaccination

Vaccine	No. survived/No. challenge inoculated		
	(% survival)		
	1 month	2 months	3 months
DP-L	4/6 (66)	2/6 (33)	3/6 (50)
BEI-DP	5/5 (100)	3/5 (60)	2/5 (40)
BEI-DPM	5/5 (100)	3/6 (50)	1/3 (33)
SEPERATED	0/8 (0)	0/8 (0)	0/8 (0)
CONTROL			
CONTACTED	Not tested	Not tested	2/2* (100)
CONTROL			

* 2 ducks that survived the outbreak were tested for immunity at the end of the project

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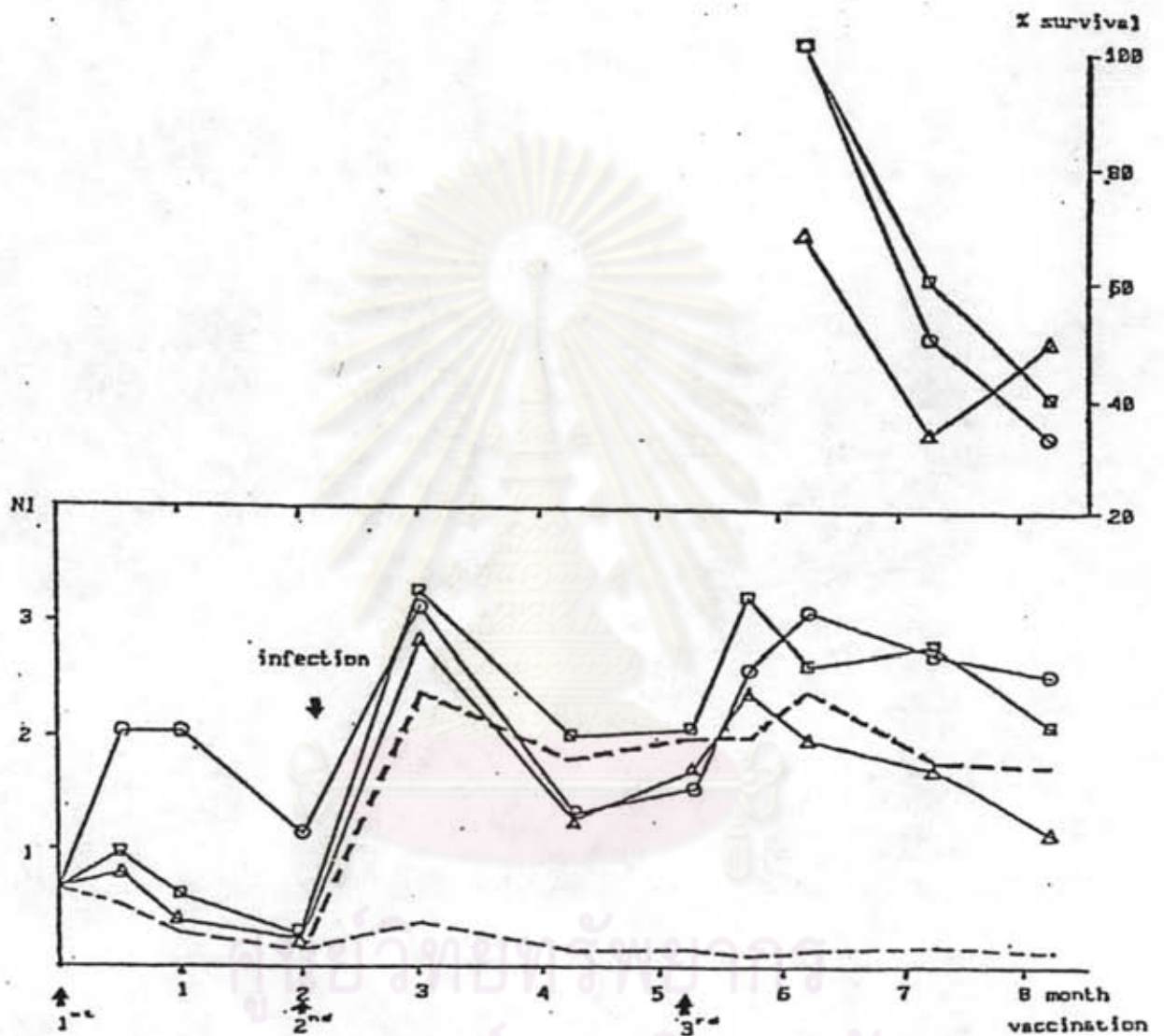


Figure 5 Comparison between NI and % survival of groups of various, vaccinated ducks from the third vaccination through the postvaccination period

100% mortality in seperated control was observed

△—△ DP-L, □—□ BEI-DP, ○—○ BEI-DPM,

----- SEPERATED CONTROL, - - - - CONTACTED CONTROL

When the NI of those vaccinated twice and three times was observed concurrently, it was found that only oil vaccine (BEI-DPM) induced an increase in titer ($p < 0.5$), while the other two vaccine did not induce any development at all ($p > 0.5$). Unexpectedly, the protective immunity provided by triple vaccination was lower than that afforded by double vaccination when compared at the same point in time (Figure 6).



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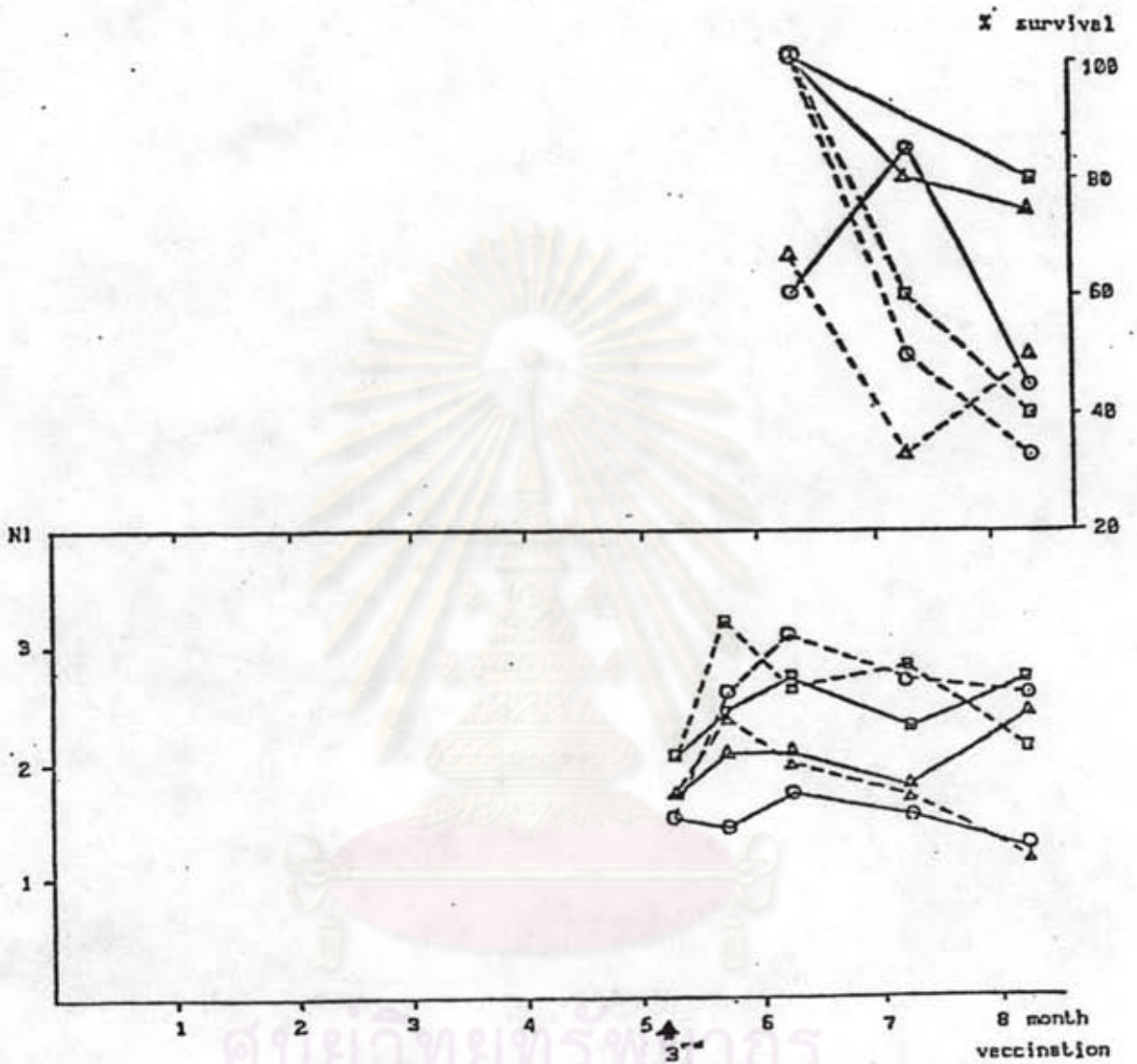


Figure 6 Comparison at the same point in time, with regard to NI and % survival between ducks vaccinated twice and three times with various vaccines

△—△ DP-L	} twice	△--△ DP-L	} three times
□—□ BEI-DP		□--□ BEI-DP	
○—○ BEI-DPM		○--○ BEI-DPM	

A challenge inoculation with virulent duck plague virus increased the NI of every duck in all groups to a high degree. A lack of correlation between neutralizing antibody titers and resistance against a challenge inoculation with virulent virus was observed during the present experiment. (Tables 8,9,10)



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Table 8 Neutralization index ($\bar{X} \pm SD$) before and after challenge with virulent duck plague ; virus relationship to protective immunity after the first vaccination

Vaccine	time after 1 st vaccination	NI (No. Serum tested)		Result of challenge	
		pre challenge	post challenge	No. of ducks survived/infected	%survival
DP-L	2 weeks	0.83±0.43 (29)	Not tested	8/8	100
	1 month	0.41±0.33 (34)	3.74±0.60 (7)	7/8	87.5
	2 months	0.22±0.21 (34)	4.28±0.43 (6)	6/7	85.7
	3 months	3.98±0.55 (4)	3.9±0.37 (4)	4/4	100
BEI-DP	2 weeks	0.97±0.62 (30)	Not tested	4/8	50
	1 month	0.63±0.52 (35)	3.93±0.81 (3)	3/7	42.8
	2 months	0.30±0.26 (30)	4.22±0.33 (2)	5/7	71.3
	3 months	3.8±1.41 (2)	3.0±0.70 (2)	2/2	100
BEI-DPM	2 weeks	2.06±1.07 (26)	Not tested	6/8	75
	1 month	2.05±1.05 (31)	3.78±0.49 (6)	6/8	75
	2 months	1.13±0.76 (31)	3.96±0.65 (3)	3/7	42.8
	3 months	3.50±0.40 (4)	3.3±0.42 (2)	2/4	50
CONTROL	2 weeks	0.11±0.23 (26)	-	0/8	0
	1 month	0.30±0.26 (34)	-	0/9	0
	2 months	0.13±0.16 (31)	-	0/4	0
	3 months	0.37±0.16 (26)	-	0/8	0

Table 9 Neutralization index ($\bar{X} \pm SD$) before and after challenge with virulent duck plague virus ; relationship to protective immunity after the second vaccination

Vaccine	time after 2 nd vaccination	NI (No. Serum tested)		Result of challenge	
		pre challenge	post challenge	No. of ducks survived/infected	% survival
DP-L	1 month	2.84±0.86 (29)	2.63±0.72 (8)	8/8	100
	2 months	1.29±0.75 (34)	3.16±1.22 (5)	5/6	83.4
	3 months	1.74±0.78 (23)	2.1±0.53 (4)	4/5	80
	4 months	2.13±0.88 (11)	2.54±0.84 (5)	5/5	100
	5 months	1.79±0.91 (10)	3.37±1.67 (4)	4/5	80
	6 months	2.43±1.10 (3)	2.8±1.65 (3)	3/4	75
BEI-DP	1 month	3.25±0.77 (23)	3.18±0.59 (5)	5/5	100
	2 months	1.98±0.78 (24)	3.03±0.90 (3)	3/6	50
	3 months	2.07±0.83 (20)	Not tested	-	-
	4 months	2.78±0.59 (8)	2.45±0.44 (4)	4/4	100
	5 months	2.38±1.02 (4)	Not tested	-	-
	6 months	2.77±0.73 (4)	2.70±0.98 (4)	4/5	80
BEI-DPM	1 month	3.12±0.64 (27)	3.02±0.86 (5)	5/7	71.4
	2 months	1.35±0.75 (34)	2.98±0.76 (7)	7/8	87.5
	3 months	1.56±0.69 (22)	2.83±0.83 (5)	5/6	83.4
	4 months	1.75±0.92 (11)	2.5±1.08 (3)	3/5	60

Table 9 Neutralization index ($\bar{X} \pm SD$) before and after challenge with virulent duck plague virus ; relationship to protective immunity after the second vaccination (continued)

Vaccine	time after nd vaccination	NI (No. Serum tested)		Result of challenge	
		pre challenge	post challenge	No. of ducks survived/ infected	% survival
	5 months	1.57±1.14 (11)	3.3±0.74 (6)	6/7	85.7
	6 months	1.26±0.94 (6)	4.16±0.94 (3)	3/6	50
SEPERATED	1 month	0.37±0.26 (26)	-	0/5	0
CONTROL	2 months	0.14±0.33 (18)	-	0/6	0
	3 months	0.12±0.23 (21)	-	0/6	0
	4 months	0.16±0.24 (13)	-	0/8	0
	5 months	0.19±0.18 (8)	-	0/8	0
	6 months	0.13±0.35 (8)	-	0/8	0

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Table 10 Neutralization index ($\bar{X} \pm SD$) before and after challenge with virulent duck plague virus ; relationship to protective immunity after the third vaccination

Vaccine	time after 3 rd vaccination	NI (No. Serum tested)		Result of challenge	
		pre challenge	post challenge	No. of ducks survived/infected	% survival
DP-L	1 month	2.02±0.55 (13)	2.83±1.37 (4)	4/6	66.6
	2 months	1.75±0.65 (10)	2.25±0.63 (2)	2/6	33.3
	3 months	1.18±0.79 (6)	3.63±0.23 (3)	3/6	50
BEI-DP	1 month	2.66±0.92 (10)	3.48±0.74 (5)	5/5	100
	2 months	2.83±0.44 (9)	3.06±0.11 (3)	3/5	60
	3 months	2.12±0.45 (5)	2.2±0.70 (2)	2/5	40
BEI-DPM	1 month	3.11±1.42 (9)	2.94±0.42 (5)	5/5	100
	2 months	2.78±1.14 (9)	4.06±0.20 (3)	3/6	50
	3 months	2.6±0.36 (3)	2.9±0.00 (1)	1/3	33.3
SEPERATED	1 month	0.16±0.24 (13)	-	0/8	0
CONTROL	2 months	0.19±0.18 (8)	-	0/8	0
	3 months	0.13±0.35 (8)	-	0/8	0
CONTACTED	-	1.8±0.6 (2)	2.5±1.0 (2)	2/2	100
CONTROL					

The association between α and β neutralization

Serums were quantified by neutralization test both α and β method in all of the serums. Parallel titration of specimens tested by these two methods were shown in Figure 7. Each point represented average titer of group of serums collected at the same time from the same vaccine. Regression equation and correlation coefficient were $Y = 1.75 X - 0.54$ and $r = 0.9556$, respectively where X was the NI (α neutralization), Y -the β neutralization titer, and r -the correlation coefficient. As foreseen, the two measurements were closely related with very small variation. From the regression line, at antibody level of NI 1.75 that signified infection was equivalent to 1:5.6 in the β method.

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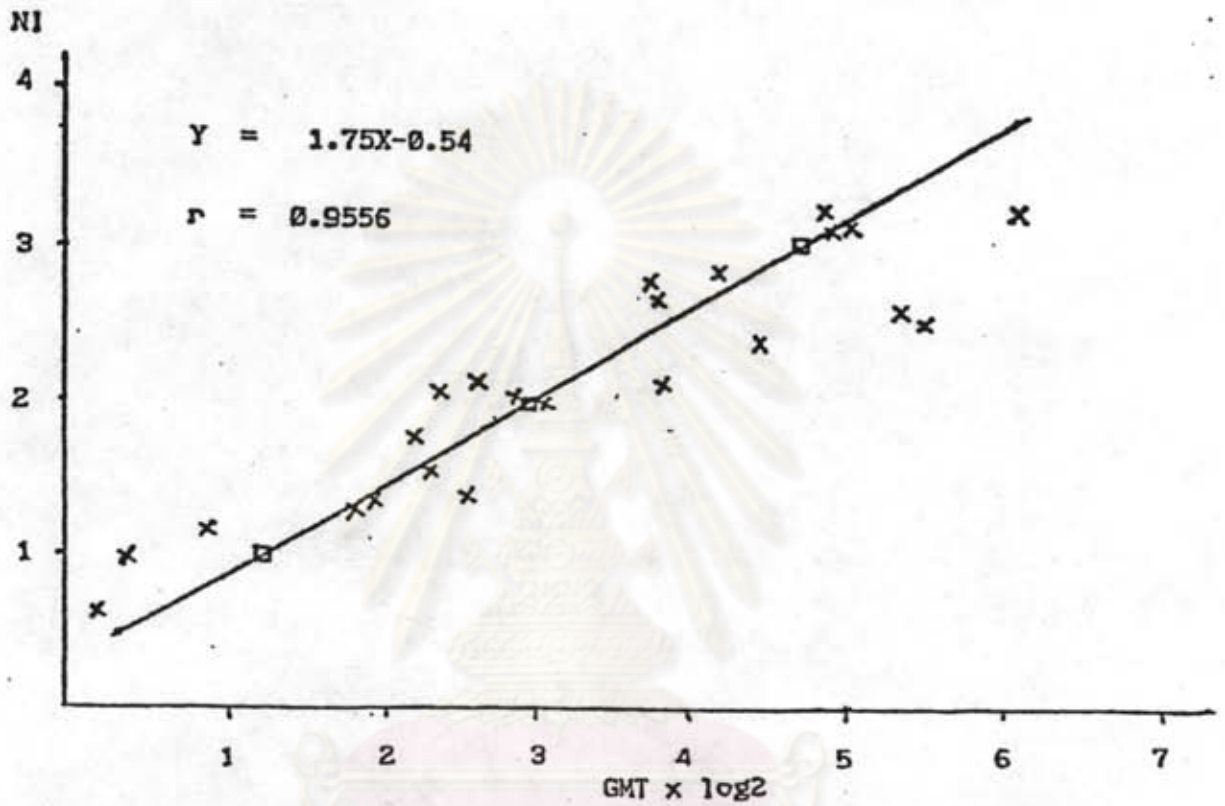


Figure 7 Scatter diagram and regression line for titer of duck serums tested by α and β neutralization test

α neutralization express as neutralization index

β neutralization express as log 2 of reciprocal of highest dilution of serum causing neutralization of infectivity

Indirect hemagglutination test (IHA)

Determination of protein content

Duck plague virus antigen contained protein 9.45 mg/ml when bovine serum albumin was used as the standard protein.

Optimal concentration of duck plaque antigen to sensitize fixed, tanned cell

The factor controlling the sensitivity of the test was the concentration of antigen. As seen in table 11 that the maximum titer from positive control, and the lowest level from negative control with no nonspecific agglutination was obtained when antigen at a concentration of approximately 23.6 ug/ml was mixed with an equal volume of 2% tanned cell in a PBS pH 7.2 at 37 °C.

Effect of duration of sensitizing

The titer did not change as the incubation time increased from 30-120 minutes (table 12). Thus, the time used to sensitize in the present experiment was 60 minutes for convenience.

Subsequent sensitizing of tanned cell was carried out with antigen (23.6 ug/ml) at 37 °C for a period of 60 minutes.

Effect of various cell concentrations on IHA patterns and titers

Duck plague virus antigen-sensitized tanned-formalinized sheep cells were suspended at various concentrations (0.5-1.5%) and added to serial dilutions of serum diluted in V-shaped microtiter plates to find the most suitable concentration. The most distinct pattern in term of sensitivity without nonspecific agglutination occurred with a 1% cell concentration. The results of which are shown in table 13.

Table 11 Effect of concentration of duck plague antigen on sensitization

duck plague antigen used for sensitization (ug/ml)	reciprocal of the end point dilution	
	positive control	negative control
9.5	128-256	<4
11.8	256-512	<4
15.7	256-512	<4
23.6	2048-4096	<4
47.3	256-512	<4
94.5	nsp	nsp

nsp = non specific agglutination of sensitized cell

Table 12 Effect of exposure time on duck plague virus sensitizing

Time of exposure 23.6 ug/ml duck plague antigen (min)	reciprocal of the end point dilution	
	positive control	negative control
30	2048-4096	<4
60	2048-4096	<4
120	2048-4096	<4

Table 13 Variation in duck plague antibody with concentration of sensitized sheep cells

Concentration of Sensitized cell (%)	reciprocal of the end point dilution	
	positive control	negative control
0.5	nsp	nsp
1	2048-4096	<4
1.5	512-1024	<4

nsp = non specific agglutination of sensitized cell

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The association between IHA and α neutralization (NI)

Figure 8 shows the correlation between the relative potencies as estimated by the IHA and α neutralization tests. Each value represents the mean titer that was calculated from the group of serums collected at the same time from the same vaccine. The regression equation was $Y = 2.80 \cdot X + 2.19$ where X was the neutralization index and Y was the IHA titer and the correlation coefficient was 0.7263. There was also a positive correlation between these two tests, although the correlation coefficient was slightly lower than the association between both types of neutralization. Also, from the regression line, at NI 1.75 that was significant level was equivalent to 1:128 in the IHA test.

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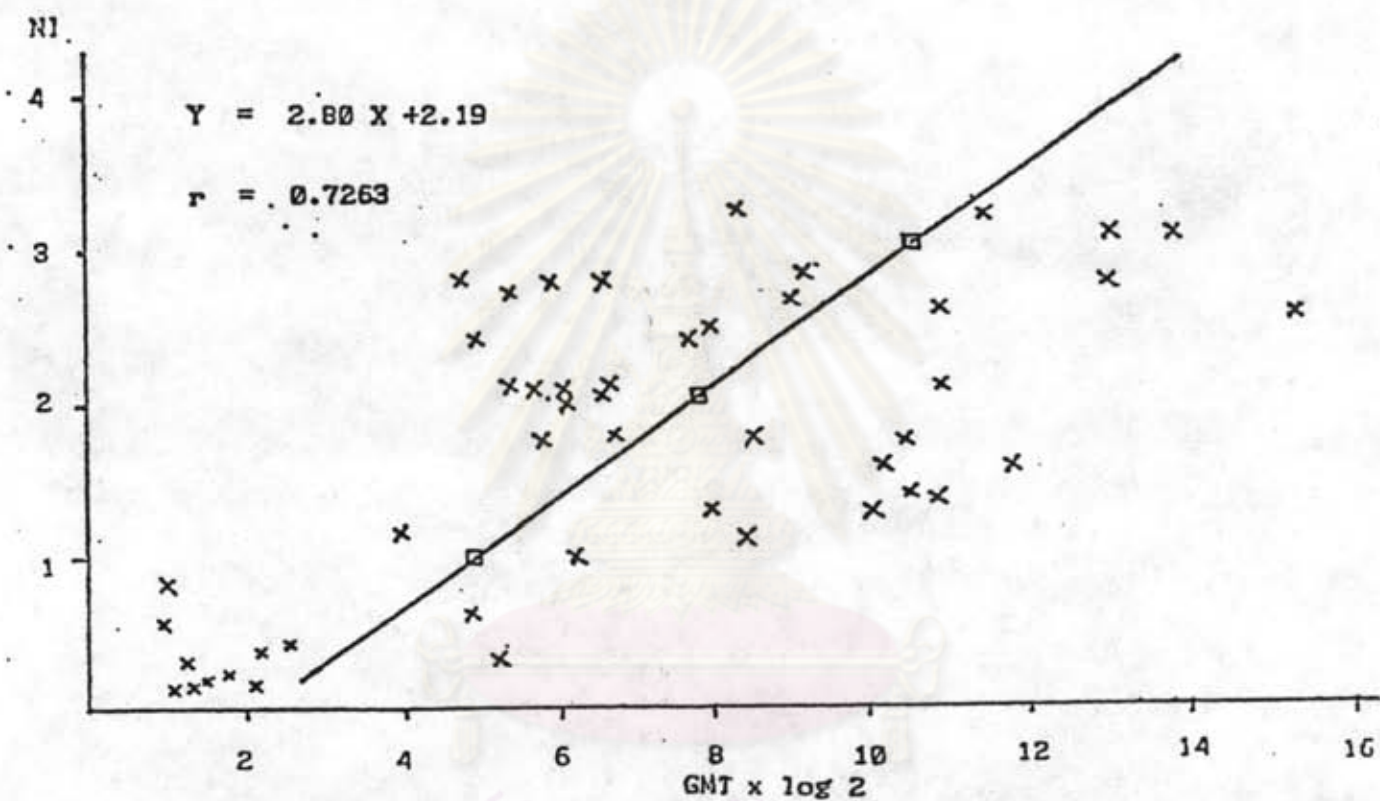


Figure 8 Scatter diagram and regression line for titer of duck serums tested by α neutralization and indirect hemagglutination test α neutralization express as neutralization index indirect hemagglutination express as log 2 of reciprocal of highest dilution of serum giving a definite agglutination