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SEROLOGICAL STUDIES ON THE IMMUNE RESPONSE AGAINST DUCK HEPATITIS VIRUS VACCINE.

(With 4 Figures and 4 Tables)

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(Received at 26/10/1992)

دراسة سيرولوجية على المناعة المكتسبة فى البط نتيجة للتحصين بلقاح الالتهاب الكبدى الوبائى

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تم اخذ حوالى ٤٠ عينة سيرم بط بيكينى محصن بلقاح بجرعة واحدة أو بجرعتين من لقاح الالتهاب الكبدى الوبائى واوضحت الاختبارات السيرولوجية المختلفة وهى اختبارات التلازن الدموى الغير مباشر - اختبار القوة المتعادلة للسيرم وكذلك اختبار ترسيب الاجار النتائج التالية : كانت من انسب التخفيفات التى تم استخدامهامن الاحماض المختلفة التى تم استعمالها فى اختبار التلازن الدموى الغير مباشر هى ١ : ٢٠٠ر٠٠٠ فى كل من حامض التنيك ومادة الكروميك كلورايد أما حامض الجلوتاميك فكان أنسب تخفيف له هو ١ : ١٠٠ وأظهرت النتائج ان مادة الكهروميك كلورايد اعطت أعلى معدل لقياس كمية الاجسام المناعية حيث وصلت النسبة الى ١ : ٢٥٦٠٠ ، ١ : ١٢٨٠ فى عينات السيرم المأخوذة من البط المحصن بجرعة واحدة أو بجرعتين على التوالى بينما كانت النسبة ١ : ١٢٨٠ ، ١ : ١٦٠ فى حالة استخدام مادة حامض التنيك وكانت ١ : ٣٢٠ ، ١ : ٨٠ فى حالة استخدام حامض الجلوتاميك (جلوتر الدهايد)فى عينات السيرم المأخوذة من البط المحصن بجرعة واحدة وجرعتين بلقاح التهاب الكبدى الوبائى على التوالى. أظهرت نتائج اختبار القوة المتعادلة للسيرم ان معاملات التعادل المصلى كانت أعلى فى عينات السيرم التى تم أخذها من البط المحصن بجرعتين حيث وصلت الى ٤ - ٧.٣ (لوج ١٠) بينما وصلت الى ١٠٢ - ٦.٣ فى العينات التى اخذت من البط المحصن بجرعة واحدة. المحاولات التى تمت باستخدام اختبار الترسيب فى الآجار أظهرت ايجابية فى العينات التى أعطت معاملات تعادل مصلى عالية بينما العينات التى كانت معاملات التعادل المصلى لها تتراوح بين ١٠٢ - ١٠٧ (لوج ١٠) أعطت نتائج سلبية. أظهرت النتائج التى أجريت باستخدام بلازما بروتين البط البيكينى المحصن بلقاح الالتهاب الكبدى الوبائى باستعمال جهاز الالكتروفروسيس لايجاد علاقة بين مادة الجاماجلوبيولين ومستوى الأجسام المناعية فى عينات السيرم وجد ان هناك علاقة متوازنة بين مادة الجاماجلوبيولين ونتائج اختبار كل من التلازن الدموى الغير مباشر والقوة المتعادلة للسيرم حيث كان هناك ارتفاع ملحوظ فى كمية البروتين وجاماجلوبيولين فى عينات السيرم المأخوذة من البط المحصن بجرعتين بلقاح التهاب الكبد الوبائى.

IMMUNE RESPONSE & DUCK HEPATITIS VIRUS VACCINE

SUMMARY

Forty serum samples were screended from vaccinated white pekin ducks for the duck hepatitis virus (DHV) antibody level after they had received a single dose or two doses of the vaccine. The results of serological examination using indirect haemagglutination test (IHA), serum neutralization test (SNT) as well as agar gel precipitation test (AGP) revealed that: (1) For the IHA test the purified and concentrated infected embryo antigen of DHV was adsorbed to sheep red cell using one of 3 sensitizing agents at different dilutions. Preliminary tests on sera of unvaccinated and experimentally vaccinated ducks showed that the best dilution of either tannic acid and chromic chloride was 1:20 000 for desensitization while that of gluteraldehyde was 1:100. Chromic chloride was also found to be the best of the 3 agents while gluteraldehyde was the least one. The antibodies in the sera of ducks vaccinated twice were found by the IHA test to be of higher titers than those in the sera of ducks vaccinated once. (2) The DHV neutralizing antibodies could be detected in the vaccinated duck sera using the SNT. The neutralization indices (NI) were higher in flocks which were vaccinated with two doses of the vaccine as it reached 4 - 7.3 whereas in case of ducks vaccinated with single dose the NI was 1.2 - 6.3. (3) The AGP test gave clear precipitating lines when testing sera of high antibody level while sera of NI ranging from 1.2-2.7 failed to produce these lines against DHV antigen. (4) The agarose electrophoresis of the plasma protein of unvaccinated and vaccinated ducks showed a correlation between the gammaglobulin and antibody titer detected by IHA and SN tests.

INTRODUCTION

Duck hepatitis virus causes a highly contagious acute fatal disease of young duckling producing about 100% mortality within 3-4 days in flocks of duckling up to two weeks old. Since the recognition of the disease and its viral etiology by *FABRICANT (1950),* there have been a number of reports on

lesions and methods for control of the disease. The most practical means of controlling infectious hepatitis in duckling appears to be vaccination of parents; a high level of passive immunity in offspring would require two doses of an attenuated virus vaccine administered to parents at least six weeks apart *(BART, 1969)*.

TAYLOR and HANSON (1967) used an IHA test for demonstration of DHV antibodies. *MURTY and HANSON (1961)* as well as *SAVIC (1967)* reported the successful application of AGP test for detecting DHV antigen in the liver and amino-allantoic fluid (AAF) of exprimentally infected chicken embryo. Many efforts were done for the development of other serological methods such as the fluorescent antibody (FA) technique *(VERTINSKI et al., 1968 and TOTH & NORCROSS, 1980)*. In the present work attempts were made utilizing the IHA to study the immune response of the vaccinated duck with DHV vaccine using a partially purified DHV antigen and either tannic acid, chromic chloride or gluctaraldehyde as a sensitizing agent for sheep erythrocytes (RBCs).

Trials have been made to utilize the AGP test and IHA test, along with the SNT for screening some vaccinated duck flocks for DHV-serum antibodies. The appearance of precipitin and the results of the IHA and NST were correlated to the electrophoresis mobility and the concentration of the plasma protein collected from vaccinated ducks.

MATERIAL and METHODS

1- Fertile chicken eggs, for antigen preparation, virus titration and SNT test, were obtained as one - day - old eggs from the General Poultry Company. The eggs were incubated for 5-10 days before being inoculated.

2- Duck hepatitis virus strain: A modified egg adopted mild living virus derived from viral hepatitis in the gooslings, attenuated on chicken embryo (SPF). The virus was obtained from IFFA MERIRUX and had been propagated twice in 9-11 days old embryonated chicken eggs (ECE); the titer was $10^{3.8}$/0.1 ml. It was preserved in 1 ml ampoule at - 20°C to serve as a stock virus and used in SNT.

3- Preparation of DHV antigen: This was carried out according to the technique of *TOTH (1969)*. A paratially purified and concentrated infected whole embryo antigen, prepared from infected embryo with the DHV strain was treated with 20% chloroform and concentrated to about 1/10 of its original volume by dialysis against polyethylene glycol (M 4000). It was used for IHA and AGP test.

4- Virus titration: It was carried out according to *ANON (1971)*, the EID 50 was calculated using the method of *REED and MUENCH (1938)*.

5- Preparation of positive control antiserum against DHV: The positive serum was prepared in susceptible DHV pekin duckling. Seven, one - day - old ducklings were obtained from the General Poultry Company Organization, El-Pasatine farm and were reared in an isolated place till four weeks. Sera were collected before vaccination to be sure that they were susceptible. Six ducklings were injected with two successive injections of 0.5 ml of DHV vaccine (EID 50 $10^{8.3}$/0.1 ml) administered subcutaneously (S/C) at least 21 days apart, one duck was kept as unvaccinated negative control. Ten days post second dose all ducks (the vaccinated and the unvaccinated control one) were slaughtered and blood was collected individually. The prepared sera were inactivated at 56°C for 30 minutes and preserved at - 20°C until used as both positive and negative serum.

6- Duck sera samples: Blood samples were obtained from 40 apparently healthy vaccinated ducks from Bahteem farm. 20 of these samples were taken 4 weeks post vaccination from vaccinated ducks which received one dose of DHV vaccine at age of two months. The other 20 were taken two months post vaccination from vaccinated ducks which received two doses of the vaccine at age of four months. All the sera were inactivated at 56°C for 30 minutes then preserved at -20 until used in different serological tests.

7- Serum neutralization test (SNT): It was carried out according to *HWANG (1969)* using constant serum - variable virus procedure.

8- Agar gel precipitation test: It was carried out following the technique described by *PANDY and SINGH (1972)* with some modification for screening duck sera for precipitating antibodies; Tween 80 was added to the antigen to accelerate the diffusion of the antigen through the agar medium and the agar plates were incubated at 38°C in a humid chamber. Each set consists of one central well filled with the partially purified DHV antigen and six peripheral wells were filled with the tested sera collected from either experimentally or field vaccinated ducks.

9- Indirect haemagglutination test (IHA): It was carried out using the microtiter technique. One of three different chemicals were used as a sensitizing agent for sheep RBCs in

the development of IHA; tannic acid, chromic chloride and gluteraldehyde.

a) Development of IHA test using tannic acid: It was carried out according to the method described by *TAYLOR and HANSON (1967)* using tannic acid in dilutions of 1:10 000, 1:20 000, 1:40 000 and 1:80 000 in phosphate buffer (PBS), pH 7.2.

b) Development of IHA test using chromic chloride: It was carried out according to *EL-NAGDY (1991)* using chromic chloride in dilutions of 1:10 000, 1:20 000, 1:40 000, and 1:80 000 in PBS pH 7.2.

c) Development of IHA test using gluteraldehyde: It was carried out according to *EL-NAGDY (1991)* using gluteraldehyde in dilutions of 1:100, 1:200, 1:400 and 1:800 in PBS pH 7.2.

10- Calorimetric determination of total protein and agar gel electrophoresis: These were performed on individual and equal volumes serum pools, collected from vaccinated one month post single vaccination and two months post two doses of vaccination and unvaccinated negative duck sera was used as negative control serum. Calculation of total protein is based on the principle of the Biuret reaction (copper salts in alkaline medium) as the method adopted by *WEICHSELBAUM (1946).* Agarose Gel Electrophoresis was run on frozen serum within two months of freezing according to methods adopted by *CARLESTROM and JAHANSSON (1983).* Briefly, 1% suspension of agarose in barbitol sodium barbital buffer 0.075 mmol/1 pH 8.6 optional inclusion of 2 ml mol/1 calcium lactate. The fractionation was carried out at potential gradient of v/cm for about 30-60 min., then transferred to the picric acid - acetic acid fixing solution for 15 min.

RESULTS

Table (1) summarized the results of the development of IHA using different dilutions of tannic acid, chromic chloride and gluteraldehyde. It also showed the results of neutralization indices along with the results of the AGPT. It was found that four samples out of six showed IHA titer of 256, when chromic chloride was used in dilution of 1:20 000 while dilution of 1:40 000 or 1:80 000 gave IHA titer of 32. Three samples out of six gave IHA titer of 32 when gluteraldehyde was used in dilution of 1:100 while the titer was 16 with dilution of 1:200. In case of tannic acid the dilution of 1:20 000 gave a titer of 256, while dilution of 1:10 000 the titer reached 128. The negative control serum which was taken from an unvaccinated duck gave a titer ranging from 0 to 2 with all different dilutions of the three sensitizing agents.

The results of neutralizing indices ranged from 4.6 to 6.5 log10EID50 and the control negative serum of unvaccinated duck had NI 1.5. All positive control sera gave positive AGP precipitin lines and the negative control serum of unvaccinated duck showed no lines of precipitation as in fig 1.

Table 2 showed the results of 3 serological tests used to examine sera of vaccinated ducks which received a single dose of the vaccine and these sera were collected after four weeks. The highest IHA titer reached 1280 in case of chromic chloride while in case of tannic acid and gluteraldehyde the titers were 320 and 160 respectively. The neutralizing indices ranged from 1.2 to 6.3. The AGP test showed two lines of precipitation with all tested sera that had NI ranging from 4 to 6.3 while those that showed NI ranging from 3.2 to 1.2 gave no lines as in fig. 2.

Table 3 showed the results of 3 serological tests used to examine sera of ducks which received two doses of the vaccine and their sera were collected two months post vaccination. The IHA titer reached 1:2560 in case of chromic chloride and 1:1280 and 1:160 after using tannic acid and gluteraldehyde respectively. The neutralization indices ranged from 4 (lowest NI titer) to 7.3 (highest NI titer). As regards the AGP test all the tested sera gave two lines of precipitation as in fig. 2.

Table 4 summarized the electrophoretic determination of 1, Equal volume pools of vaccinated pekin ducks collected either one month post a single vaccination or two months post the second dose of vaccination, 2, Individual samples of either types of ducks as well as a serum sample of an unvaccinated duck.

Fig. 3 revealed the electrophoretic profile of individual and pooled samples of sera of vaccinated ducks as well as a control unvaccinated duck. The area under the peak was integrated.

Fig. 4 showed the agarose electrophoresis of plasma protein of unvaccinated and vaccinated ducks.

DISCUSSION

The most practical means of controlling infectious hepatitis in duckling is vaccination of flocks *(ASPLIN, 1956)*. Few reports are available in the literature on the development and practical application of the serological procedures for the detection of DHV antigen and serum antipodies using IHA and AGP tetes. In the present investigation attempts were made to apply

IHA and AGP tests as well as SNT for screening ducks for DHV serum antibodies. as regards the IHA test trials were made using different sensitizing agents (tannic acid, chromic chloride and gluteraldehyde) with different dilutions in order to choose the best and more sensitive sensitizing agent with an optimum dilution that is able to detect DHV antibodies in sers of experimentally vaccinated ducks as compared with serum of unvaccinated ducks.

The experiments revealed that the best dilution of either tannic acid or chromic chloride was 1:20 000 for sensitizing red cells for the adsorption of DHV while that of gluteraldehyde was 1:100. The results also showed that chromic chloride was the best sensitizing agents since it was able to demonstrate high titers in sera of vaccinated ducks while gluteraldehyde was the lest sensitive agent. Partially purified and concentrated whole embryo antigen of DHV was used to demonstrate IHA antibodies in immune duck sera when tannic acid or chromic chloride was used as asenitizing agent. *MURTY (1960) and WCHENDOERFER (1965)* Were unable to demonstrate IHA antibodies in immune duck sera when unpurified DHV antigen was used.

TAYLOR and HANSON (1967) used a partially purified antigen concentrated by (NH4) SO4 and reported that the antigenic preparation of DHV should be needed in order to give positive reaction in IHA test as had been previously reported by *BOYDEN (1951).*

Our results revealed that IHA antibodies were higher in serum that were collected from vaccinated ducks with two doses of DHV as the titer was double that collected 4 weeks post vaccination from ducks receiving single vaccination of DHV vaccine.

BART (1969) revealed that a high level of passive immunity in offsprings would require two doses of an attenuated virus vaccine administered to parent stock at least six weeks apart. Immunity was adequate for about 9 months after second vaccination. *CHETH (1979)* proved that the hyperimmune sera collected 20 days after the last antigenic stimulus with DHV revealed higher titers. The author also reported that IHA test proved to be sensitive in detecting DHV antibodies as it could be demonstrated in variable incidence and levels in the sera of vaccinated ducks including day-old-duckling but the highest titer in sera from about 16 week-old-breeding duck that have been vaccinated with live DHV vaccine 4 weeks before blood colection.

As regards the AGPT in this study precipitating antibodies could be detected in all duck sera with high levels of IHA and

NI antibodies while it could not be detected in sera of low antibody level as shown in five sera that gave low NI. Two lines of precipitation could be detected when using sera of experimentally or naturally vaccinated duck sera while no such lines could be seen in the sera of unvaccinated ducks.

MURTY and HANSON (1961) conducted microgel diffusion and found lines of precipitation which appeared within 4 to 24 hours and could be observed by indirect light. When hyperimmune rabbit serum was used, 3 lines of precipitation were observed while with duck immune serum sample only a single line of precipitation appeared after 24 hours.

CHETH (1979) found one or two precipitation lines which developed after single filling of the reactants when hyper immune rabbit serum was examined against different DHV antigen. Hyperimmune duck serum gave a single precipitation line after 2-3 refillings of the reactants when examined against infected antigen preparation. On the other hand, *WACHENDOERFER (1969)* failed to demonstrate virus specific precipitating line by AGP test. *TOTH and NORCROSS (1980)* found that none of numerous duck anti-DHV immune sera, with virus neutralizing activity in the range of 1.8 5.57 log10 EID50 NI, developed precipitation lines against a variety of DHV preparations tested in low, and high ionic strength agar. They concluded that AGP test was unsuitable for serologic activity of duck sera for anti- DHV antibody activity.

Our results revealed that the serum neutralization indices ranged from 1.2 to 6.3 log10 (EID50) of virus neutralized per 0.1 ml of serum colected 4 weeks post vaccination from ducks which received one dose of vaccination. A considerable increase in the neutralizing indices was noticed, 4 to 7.3 log10 (EID50) of virus neutralized per 0.1 ml., of serum colelcted 2 months post vaccination. Eventually, the second dose of the vaccine acted as a boaster and helped in rising the NI of vaccinated ducks.

HWANG (1972) found that the NI ranged between 3.5 up to 6.2, 14-35 days post vaccination. *LEVINE (1972)* revealed that ducks surviving infection with virulent field DHV responded with high VN antibody development, and titers ranging from 10 to 10 could be found in 4 to 8 weeks old market ducks originating from infected premises.

CHETH (1979) conducted SNT on serum of vaccinated flocks at age of 16 weeks old and found that 39.5% of samples proved to contain neutralizing antibodies. The author also found that in flocks where IHA antibodies could be detected, neutralizing antibodies were also demonstrable.

NAWWAR (1980) revealed that there was a gradual increase in neutralizing indices starting from 0.9 up to 6.2 log10 (EID50) in different occasions during 4-34 days observation period after vaccination.

The results shown in this study revealed that the changes in the gamma - globulin reflected the response of the reticulo - endothelial system to vaccinal DHV antigen and these appeared to be a correlation between the concentration of gamma - globulin and antibody titers. The marked increase in the concentration of total serum protein and gamma - globulin after two doses of DHV and also the changes in the gamma - blobulin were roughly parallel with the serum neutralizing indices and IHA titers.

In conclusion, on comparing the results of the 3 tests, the IHA test was proved to be the simplest and rapid method for screening duck sera for DHV antibodies. On the other hand, the SNT requires a considerable time, materials and equipment. The AGP test although it is a rapid simple serological procedure yet it doesn't give an accurate results being unable to determine the level of immunity in vaccinated ducks.

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Table (1) Examination of sera from 6 experimentally vaccinated pekin ducks and a negative control one by 3 serological tests.

Positive control serum sample No.	Titer of indirect haemagglutination test dilution of *												SNT	AGP
	Tannic Acid				Chromic Chloride				Gluteraldehyde					
	1	2	3	4	1	2	3	4	1	2	3	4	NI	Test
1	32	128	16	16	128	256	16	16	16	16	4	2	5	+ ve
2	16	64	8	8	64	64	32	8	8	8	4	2	4.6	+ ve
3	64	128	32	16	128	256	32	16	32	16	2	2	5.6	+ ve
4	128	256	16	16	64	128	32	32	32	8	2	2	6	+ ve
5	64	128	16	8	64	256	16	16	16	8	4	4	6	+ ve
6	128	256	8	8	256	256	32	16	32	16	4	4	5.5	+ ve
-ve control	2	2	2	0	2	2	2	0	0	2	2	0	1.5	- ve

* Tannic acid or chromic chloride dilutions :

1 = 1 : 10 000

2 = 1 : 20 000

3 = 1 : 40 000

4 = 1 : 80 000

Glyteraldehyde dilutions :

1 = 1 : 100

2 = 1 : 200

3 = 1 : 400

4 = 1 : 800

Table (2) Examination of 20 sera of pekin ducks 4 weeks after a single dose of DHV vaccine using 3 serological tests.

No. of tasted sample	Indirect Haemagglutination Titer (IHA)*			SNT	AGP
	Tannic A. 10:20 000	Chromic Ch. 1:20 000	Gluteraldehyde 1:100	(NI)	Test
1	80	320	40	5.2	+ve
2	20	80	20	4	+ve
3	40	80	40	4	+ve
4	40	160	40	4.6	+ve
5	80	160	40	4.7	+ve
6	40	160	40	4.5	+ve
7	320	1280	160	6.3	+ve
8	160	320	80	5.2	+ve
9	160	320	80	5.4	+ve
10	40	160	40	4.5	+ve
11	80	80	20	2.7	-ve
12	10	20	0	4.47	+ve
13	20	20	0	3.4	+ve
14	40	40	10	2.5	-ve
15	40	80	20	3.2	-ve
16	10	10	0	1.2	-ve
17	20	20	10	3.5	+ve
18	80	80	40	4	+ve
19	160	160	160	5.1	+ve
20	20	20	0	2.7	-ve

* Each of the 3 sensitizing agents was used at an optimum dilution.

Table (3) Examination of 20 sera of pekin ducks 2 months post second dose of DHV vaccine using 3 serological tests.

No. of tested sample	Indirect Haemagglutination Titer (IHA)*			SNT	AGP
	Tannic A. 10:20 000	Chromic Ch. 1:20 000	Gluteraldehyde 1:100	(NI)	Test
1	160	320	80	6	+ve
2	160	160	80	5.6	+ve
3	160	160	80	5.6	+ve
4	80	80	40	4	+ve
5	1280	2560	160	7.3	+ve
6	320	320	160	6.2	+ve
7	1280	1280	160	7.2	+ve
8	1280	320	160	7	+ve
9	640	320	80	6	+ve
10	160	80	80	4.8	+ve
11	160	160	80	5.2	+ve
12	80	80	40	4	+ve
13	80	80	40	4	+ve
14	80	160	40	5.6	+ve
15	80	160	80	5.7	+ve
16	80	160	80	5.5	+ve
17	1280	2560	160	6.3	+ve
18	1280	320	80	5.2	+ve
19	1280	1280	80	5.4	+ve
20	80	160	40	4.5	+ve

Each of the 3 sensitizing agents was used at an optimum dilution.

Table (4) Electrophoretic determination of different serum samples collected from vaccinated ducks.

Components of serum	Control serum	Serum samples post vaccination			
	No. 3	No. 6	No. 1	No. 2B	No. 2
Total protein gm/100	4.000	3.400	3.500	6.800	3.300
Prealbumin	0.080	0.100	0.060	0.170	0.080
Albumin	2.310	1.630	1.210	2.900	1.620
Alpha,1,globulin	0.410	0.360	0.260	0.500	0.340
Alpha,2,globulin	0.430	0.180	0.090	0.430	0.200
Beta globulin	0.220	0.220	0.360	0.570	0.270
Gamma globulin	0.550	0.900	1.520	2.220	0.790
Albumin/Globulin Ratio	0.021	0.032	0.017	0.025	0.026

Notes on serum samples :

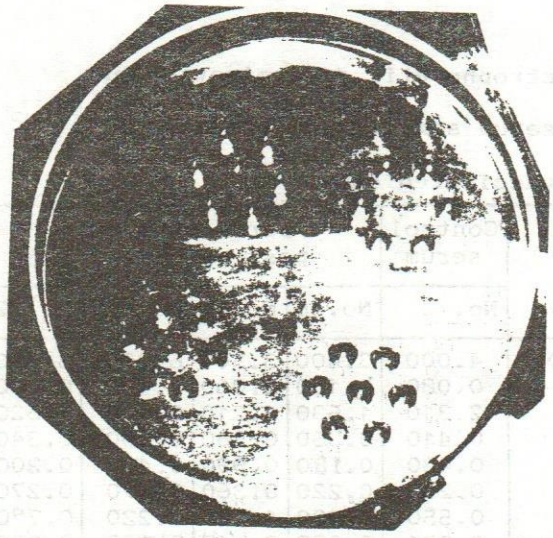
No. 3 : Individual unvaccinated duck.

No. 6 : Individual sample collected from vaccinated duck one month post vaccinated with one dose of DHV vaccine.

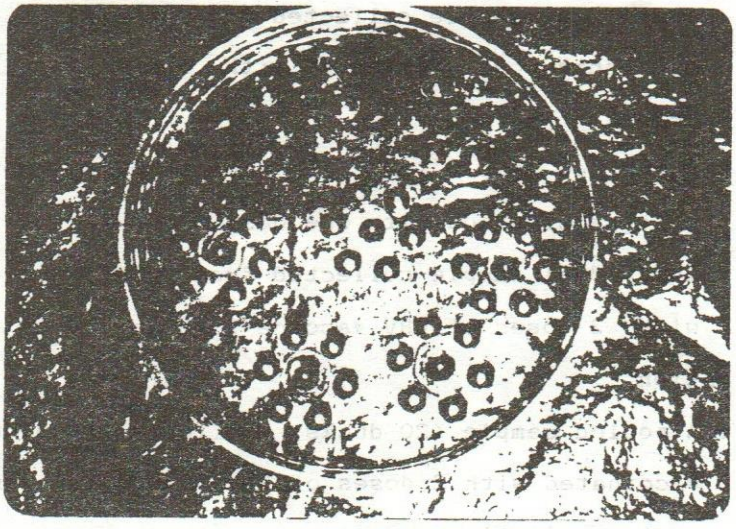
No. 1 : A pooled sample (20 ducks) collected one month post vaccination with one dose.

No. 2B : Individual sample collected from vaccinated duck with 2 doses of DHV vaccine 2 months post vaccination.

No. 2 : A pooled sample (20 ducks) collected from ducks vaccinated with 2 doses of DHV vaccine 2 months post vaccination.

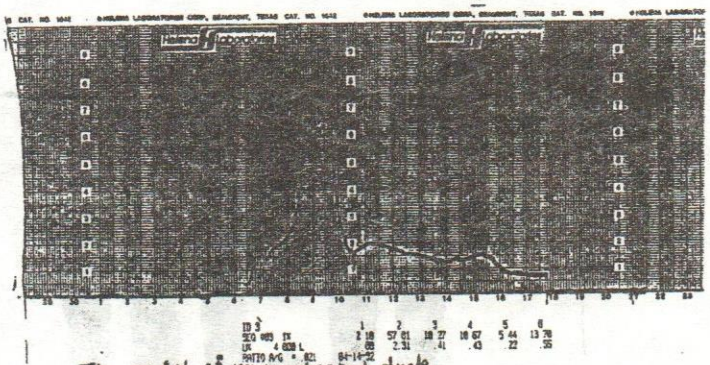


Fig(1) development of AGP test conducted on positive and negative control serum samples collected from expr-imentally vaccinated and unvaccinated duck.

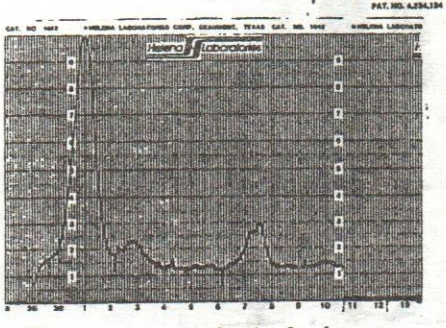


Fig(2) development of AGP test conducted on the different serum samples collected from vaccinated ducks one month and 2 months post vaccination.

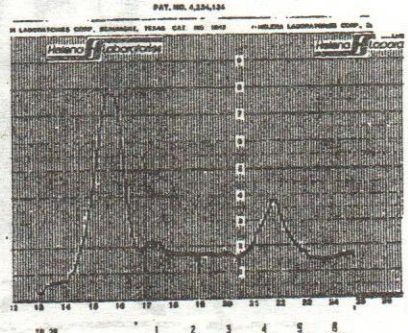
Fig. (3) The electrophoretic profile of sera collected from unvaccinated and vaccinated ducks.
The area under the peak was integrated.



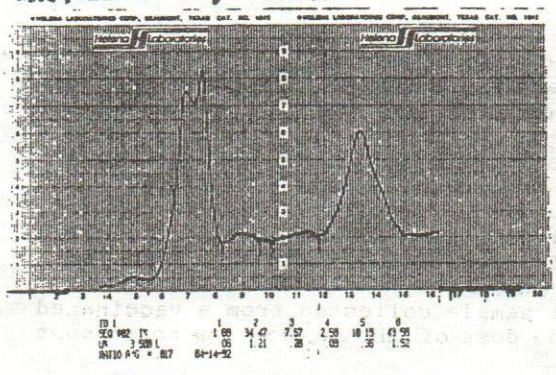
The profil of unvaccinated duck



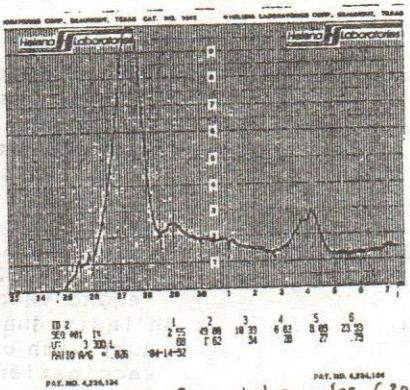
The profil of individual duck vaccinated with a single dose, serum collected after 1 month



The profil of individual duck vaccinated with 2 doses collected after 2 months.

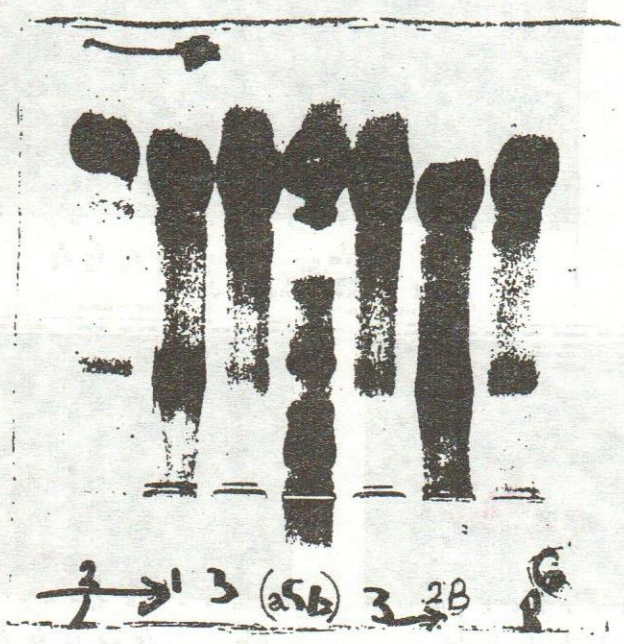


The profil of pooled samples (20 ducks) of the above type



The profil of pooled samples (20 ducks) of the above type.

Fig. (4) Agarose electrophoresis of plasma protein of unvaccinated and unvaccinated ducks.



(a & b) & (3) : unvaccinated control serum.
(2) : A pooled sample (20 ducks) collected from vaccinated ducks with 2 doses of DHV vaccine 2 months post vaccination .
(1) : A pooled sample (20 ducks) collected from vaccinated ducks with 1 dose of DHV vaccine 1 month post vaccination .
(2B) : An individual sample collected from a vaccinated duck with 2 doses of DHV vaccine 2 moths post second vaccination.
(6) : An individual sample collected from a vaccinated duck with one dose of DHV vaccine one month post vaccination.