BS. VET. MED. J. NOVEMBER 2007, 5TH SCIENTIFIC CONFERENCE, P. 79-86

Beni-Suef Veterinary Medical Journal

Immune response of broiler breeder chickens to inactivated Avian influenza H5N1 vaccine under field condition

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This study was carried out on serum samples collected from broiler breeder chicken flocks vaccinated with avian influenza (AI) H5N1 inactivated vaccine. These flocks included 23 flocks aged 13 to 47 weeks reared in close houses in 7 sites; two vaccinated breeder flocks for HI antibody monitoring by 5 weeks interval samples and 8 flocks aged 41 weeks reared in different sites with identified females and males samples. The vaccine was used in a dose of 0.2 ml at 1 day in hatchery and revaccinated with 0.5 ml at age of 18 days, 19- 20 weeks and 40 weeks. Hemagglutination inhibition (HI) test was carried out against homologous antigen.

The study pointed out that AI H5N1 inactivated vaccine under field application induced irregular and low HI titres following the 1st two doses ranged from log 2 0.0 to 4.15 with great variation between flocks, where samples with titre 0-2 ranged from 20 to 100%. The 3rd dose at 19-20 weeks was essential to elevate HI titres 3.25 to 7.44 with more homogenizes flock immunity and lower percentage of titres 0-2 (0-20 %) and as measured by HI test. Revaccination of layer flocks at 40 weeks (fourth dose) improves flock immunity facing stress of egg production as evaluated by HI (5.52 - 6.33) and lower negative percentage (5.5-11.7%). Monitoring of breeder flock every 5 weeks is essential to detect proper time of revaccination as each flock has its HI antibody curve. There was a difference in HI tit re rang log 2 0.33 to 1.2 between male and female chicks reared in the same house, but this variation not affecting flock mean.

Birds at aged 41 weeks having titres < $\log_2^3$ (Seronegative) were protected when exposed to contact with infected flock as showed no clinical signs or change in HI titres after 12 days.

In conclusion the usage of homologous inactivated H5N1 vaccine in 4 doses in layer flocks was of value in improving chicken immunity to AI H5N1 wild strain circulate in our field.

Avian influenza (AI) is a notifiable disease caused by influenza A viruses related to Family *Orthomyxoviridae* (Voyles, 2002). AI in domestic chickens and turkeys can be classify according to disease severity to severe; mortality rates in infected flocks often

approach 100%; due to highly pathogenic AI (HPAI), and asymptomatic due to low-pathogenic AI (LPAI) (Horimoto and Kawaoka, 2001).

The OIE considered AI infection of poultry caused by any AI virus of the H5 or H7 subtypes or by any AI virus with an intravenous pathogenicity index (IVPI) greater than 1.2, and countries that identify HPAI should report the occurrence to OIE within 24 hours. (OIE, 2004).

AI virus subtypes are 16 different HA antigens and 9 different NA antigens. The last one HA types (H16) had been recognized, from black-headed gulls caught in Sweden and the Netherlands in 1999 (Fouchier *et al.,* 2005).

H5N1 strains are of worldwide circulation in birds, are responsible for the current severe outbreaks in poultry, other birds, Human, feline and other mammals in Asia, Africa, Europe and USA (Keawcharoen *et al.,* 2004, Thanawong-nuwech *et al.,* 2005; Webster *et al.,* 2006).

Aquatic birds, particularly ducks, shore birds, and gulls, are considered the natural reservoirs for AI viruses (Webster *et al.,* 1992 ; FAO/OIE/WHO, 2004). These birds generally do not develop disease when infected (Horimoto and Kawaoka 2001; Webster, *et al.,* 2006); however, an outbreak of H5N1 was identified in migratory geese and other wild birds in Qinghai, China, May 2005 (Lui *et al.,* 2005) and from asymptomatic free sparrows in Henan, China (Kou *et al.,* 2005). Asymptomatically infected domestic ducks are shedding more H_5N_1 virus for longer periods (WHO, 2004).

Since 1999, the number of occurring HPAI outbreaks has increased significantly (Capua *et al.,* 2002). Outbreak of H5N1 1997 in Asian poultry in Hong Kong followed by a wide spread of the virus to poultry and humans. The outbreak

was apparently stopped by slaughtering all domestic chickens (Snacken *et al.,* 1999). The outbreak was reemerged in summer 2004 in several Asian areas and stormily spread toward Europe and Africa to reach Egypt and Nigeria in mid February 2006. This virus spread was attributed to Free ranging backyard chickens and ducks, illegal transportation of birds as well as infected migratory waterfowl (Li *et al.,* 2004; Chen *et al.,* 2005; Liu *et al.,* 2005; Tiensin *et al.,* 2005; Webster *et al.,* 2006).

Prevention of AI passed on strategies by APHIS (2002, FAO (2004) ; FAO/ OIE/WHO, (2005) as biosecurity to prevent exposure of flocks to the influenza virus; continuous monitoring; reporting of AI suspected and applying control measures; depopulation and disinfection and quarantine of positive cases as a short strategy (Stegeman *et al.,* 2004). In endemic area, vaccination of poultry flocks by inactivated or gene vaccines became the only solution in the long-term strategy. Vaccination is targeting to lower losses from mortality, reduce the viral load in the environment and risk of human infection as well as eradication of positive cases (Luschow *et al.,* 2001; FAO, 2004; OIE, 2005a; Van der Goot *et al.,* 2005).

Two different types of inactivated (homologous H determinant and heterogonous N determinant) vaccines in oil-based emulsion are available for usage by injection (FAO, 2004). Infected chickens can yield positive hemagglutination inhibition (HI) antibody as early as 3 to 4 days after the appearance of first disease signs, HI- test can be useful as a serological test for diagnosis of the disease and evaluation of immune response of vaccinated chickens with inactivated vaccine as recommended by (Allan, 1981; Beck and Swayne 1997; OIE, 2004).

In Egypt, AI was under focus as enzootic cases of fowl plague had been reported 1923-1945 (Alexander, 1986 and 1992). The production of local vaccine was continued until complete diminish of disease at 1960s -1970s; where the production and use of vaccine was stopped. in mid February 2005 outbreaks of H5N1 had reported in backyard and commercial poultry flocks with human cases. Following the failure in "stamping out" both homologous Chinese (H5N1) and heterologus (H5N2), inactivated vaccines were used for prevention of the disease.

The usage of Chinese inactivated H5N1vaccine was followed by a storm of discussion about its activity and immunogenicity. Nowadays, both vaccines are used in poultry farms with reporting of considerable number of outbreaks.

From the above mention, our study planed to evaluate field application of the used homologous AI vaccine in immunizing breeder chicken flocks and estimate the post vaccinal immunity using HI-test with special consideration to the following. i) Detection of immune response of flocks reared in different locality, given the same vaccine and vaccination system, and at the same age. ii) Comparing the immunity curve in 2 flocks by testing 5-week interval samples. iii) Comparing antibody levels in male and female birds of the same flock. iv) Ability of birds having undetected or low antibodies (considered seronegative) to contract infection in contact with naturally infected birds as a challenge.

Materials and methods

Serum samples. Random individual blood samples were collected for serum through wing vein of vaccinated chickens including:
i) Vaccinated broiler breeder chicken flocks (23 flocks) aged 13 to 47 weeks of age as mixed sex samples. These flocks were reared in close houses in 7 sites with collection of 441 samples (17-20/flock) (Table 1 Fig. 1,2). ii) Two vaccinated breeder flocks were serologically monitored for AI H5 antibody response following vaccination in two flocks at (Table 2, Fig. 3,4)
a. The 1st flock: 5th to the 50th week of age.
b. The 2nd flock: 25th to the 55th week of age.
3. Identified numbered females (10-12) and males (2-5) from 8 flocks aged 41 weeks reared in different sites (Table 3, Fig. 5). Identified birds were kept in isolated pen until HI testing.

Contact infection. Seronegative 26 chicken of flocks 8-10 aged 18 weeks (Table 1) were subjected to contact infection by transfer them to infected vaccinated house; where most of sentinel birds were died. Sera from contact birds were collected and HI tested against H5N1 antigen 12 days later.

Positive and negative sera. Both negative and positive AI HI sera supplied by H5N1 vaccine producer used as serological test controls.

Antigen. H5N1, lyophilized antigen for HI tests supplied by the vaccine producer was used. The antigen was diluted in PBS and adjusted to 4 HI units before use in evaluation of immune response (OIE, 2005).

HA and HI-tests. Methods recommended and described by OIE (2005b) were used to identify AI antigen or serological monitoring of immune

response. HI result was interoperated as recommended by CEC (1992); OIE (2005). HI-results were given titre reference number (TRN) according Kaleta and Siegmann (1971).

Vaccines. The Chinese inactivated H5N1vaccine distributed by VACSERA, Agoza, Egypt was used in vaccination of chicken flocks.

Vaccination. Chickens were injected subcutaneously with the inactivated H5N1 oil adjuvant vaccine in the neck. The vaccine was used in a dose of 0.2 ml at 1 day in hatchery and revaccinated with 0.5 ml at age of 18 days, 19- 20 weeks and 40 weeks.

Results

Results of HI test (Table 1, Fig. 1) showing that flocks aged 13 weeks (6 flocks) were having variable titres ranging from 1.3 in flock 5 to 4.15 in flock 1. Flock number 15 showed HI mean titre 2.33, while flocks aging 18 weeks showed some what lower titres ranged from 0.0 (flock 20) to 3.87 (flock 14). Flocks 16 and 17 those received the 3rd dose of vaccine showed increased HI titres 4.00 and 4.84; respectively. Flocks aged 26 weeks (18 and19) showed titres of 6.65 and 7.44 at the 7th week post 3 rd vaccination. Chicken flocks aged 39 weeks (flock 20 and21) showed decreased titres to reach 3.66 and 3.25; respectively. Chicken flocks 22 and 23;those aged 47 weeks and given the 4th dose of vaccine at the 40th week showed HI titres 6.33 and 5.52; respectively.

The repeated vaccination resulted in lowering in the percentage of birds showing titres < $\log_2$ 0-2 (Table 1 Fig.2) to be 20-100% in birds received 3 doses (flocks 1-15),0-20% in 4 and 5 doses (flocks 16-23). The percentage of negative samples according to number of vaccine doses are 3 vaccine doses 140/293 (47.78 %), 4 vaccine doses 11/113 (9.73 %) and 5 vaccine doses 3/35 (8.57 %).

Results in table 2 showing the HI titres in 5-week interval in sera of vaccinated breeder flocks:

In flock 1 (Fig. 3) the mean HI $\log_2$ titres were 5.15 at the 5th week of age (after 3 vaccination) then decreased to 3.50 at the 15th and elevated from the week 20 following the 3rd vaccination to reach the highest titre 6.25 at the week 25. The HI titres decreased to 4.45 at the 40th week of age, where the last AI vaccination which resulted in another increase in titre to 6.25 after 5 weeks.

In flock 2: The detected HI $\log_2$ titre was 7.30 at the 25 week and decreased to 4.95 at the 40th week where the titre decrease was continue after the vaccination at 40th week of age to be elevated at the 50th week to reach 6.95 (Fig. 4). HI antibody curve is different in flock 1 and 2, but in both, there is a decline phase at age of 25-40 weeks (maximum egg production).

Table (3) and Fig. (5) showed the HI titres in female and male chickens samples from 8 flocks aged 41 weeks one week following the 4th vaccination. Male chickens samples having HI titres higher than females in 6 flocks 1,2 and 5-8, while titres of males were lower in flocks 2 and 3. The difference in titres between the males and females was between $\log_2$ 0.33 (flock 1) and 1.2 (flock 8). The variation between male and female titres is not affecting the flock means. The contact birds (Seronegative) showed no clinical signs or higher levels of HI titres after 12 days contact with the infected flock.

Discussion

Avian influenza (AI) H5N1 virus strains are of worldwide circulation in birds, responsible for the current severe outbreaks in poultry, other birds, Human, feline and other mammals in Asia, Africa, Europe and USA (Keawcharoen *et al.,* 2004, Thanawongnuwech *et al.,* 2005; Webster, *et al.,* 2006). World human and animal health authorities (WHO, OIE and FAO) considered AI H5N1 as a notifiable disease required international cooperation on the scientific, information and economical to combat such infection and avoid the possible human pandemic.

Avian influenza prevention had been regular monitoring, hygienic measures to prevent infection and spread while control is based on eradication, disinfection quarantine and compensation (APHIS 2002; FAO, 2004; Stegeman *et al.,* 2004; FAO/OIE/FAO, 2005a,b).

Vaccination is targeting to lower losses from clinical signs and mortality, reduce virus shedding and environmental load and risk of both poultry and human infection with continuous eradication of positive farms (Luschow *et al.,* 2001; Swayne *et al.,* 2000; FAO, 2004; OIE, 2005a; Van der Goot *et al.,* 2005). Following the failure in stamping out both homologous (H5N1) and heterologus (H5N2), inactivated vaccines were used for prevention of the disease.

Serological testing, especially HI test is useful for evaluation of immune response of vaccinated chickens with inactivated vaccine (Allan, 1981; Beck and Swayne, 1997; OIE, 2004).

Our study to evaluate field application of the used H5N1 AI vaccine in immunizing breeder

chicken flocks by HI-test using homologous H and N antigen supplied by the vaccine producer was carried out.

Results of HI test (Table 1, Fig. 1) showing that flocks aged 13 weeks (6 flocks) having variable titres ranging from 1.3 to 4.15,while flocks aging 18 weeks showed also lower titres (0.0 to 3.87). Min *et al.,* (2004) reported similar results where HI titre for inactivated H5N1 vaccine increased 14 days post vaccination to 27.5 and maintained at 25 level on day 210 and birds were resistant to challenge 18 days post vaccination.

Flocks received the 3rd dose of vaccine (16 and 17) showed high HI titres (4.00 and 4.84) one week later, and flock 18 and 19 (6.65 and 7.44) at the 7th week), respectively. While, chicken flocks aged 39 weeks (flock 20 and 21) showed lower titres (3.66 and 3.25); respectively. These results indicated that AI vaccines resulted in lower and irregular titres as stated by Salem (1995) who reported in constant antibody titres in vaccinated chickens and ranging from none to high titres.

Chicken flocks aged 47 weeks and given the 4th dose of vaccine showed HI titres 5.52-6.33. This result showed that revaccination is important to obtain higher titres as mentioned by Stone (1987) used inactivated H5N2 vaccine in white leghorn layer chickens at 12 and 20 weeks, at 8 weeks post vaccination HI antibodies were 1/597 and protection was 90-100% and CEC (1992) who reported that birds vaccinated twice exhibited higher serological titres as compared to those vaccinated once. While Ai *et al.,* (2004) reported that the highest antibody level against H9 (average 6.72) was observed at 31-80 days of age in 10 days vaccinated chickens.

The repeated vaccination resulted in lowering in the percentage of birds showing titres < $\log_2$ 0-2 (Table 1 Fig.2). Similar result hah been reported by Swayne *et al.,* (2000) who concluded that commercial H5 AI vaccines could protect poultry from 1997 Hong Kong H5N1 strain, and the repeated vaccination is recommended for increase number of seropositive birds.

HI titres in 5-week interval sera of vaccinated breeder flocks, where in flock 1 (Fig. 3) the mean HI $\log_2$ titres were 5.15 at the 5th week of age (after 2 vaccination) then decreased to 3.50 at the 15th and elevated from the week 20 following the 3rd vaccination to reach the highest titre 6.25 at the week 25. The HI titres decreased to 4.45 at the 40th week of age, where the last AI vaccination which resulted in another increase in titre to 6.25 after 5 weeks. In flock 2: The detected HI $\log_2$ titre was 7.30 at the 25 week and decreased to 4.95 at the 40th week where the titre decrease was continue after the vaccination at 40th week of age to be elevated at the 50th week to reach 6.95 (Fig. 4). HI antibody curve is different in flock 1 and 2, but in both, there is a decline phase at maximum egg production (age 25-40 weeks). These results proved that immune response to the same vaccine was differing with the flock and the repeated vaccination is essential to maintain high titres.

HI titres in female and male chickens flocks aged 41 weeks one week following the 4th vaccination where, male chickens having HI titres generally varied from higher to lower from flock to anther. The difference in titres between the males and females was between $\log_2$ 0.33 and 1.2. The variation between male and female titres is not affecting the flock means. This point needs more studies.

The contact birds (Seronegative) showed no clinical signs or higher levels of HI titres after 12 days contact with infected flock. This results are in agreement with results of Capua *et al.,* (2002) who reported that birds having titres <1:2 and 1:4 were died 4-6 days post H7N1 challenge. While, Swayne *et al.,* (1999) reported that 41% of hi negative vaccinated chickens resist challenge and all chickens with detectable HI-titres were protected. The result can be explained by Swayne *et al.,* (1999 and 2000) where the level of protection against mucosal infection and subsequent shedding of challenge virus may depend on the degree of sequence similarity between HA gene of vaccine and challenge virus. Moreover, Brugh and Stone (1986) reported that layer chickens had protected for 30 weeks after single vaccination.

This study pointed out that AI H5N1 inactivated vaccine under field application induced irregular and low HI titres following the 1st 2 doses and the 3rd dose at 19-20 weeks was essential to elevate and homogenizes flock immunity as measured by HI test. Revaccination of layer flocks at 40 weeks improves flock immunity facing stress of egg production as evaluated by HI results and contact infection.

Table (1): HI titres against H5 in chicken flocks of different sites and ages.

Flock No	Age /w	No of samples	Distribution of HI - TRN							Mean ± SD		% 0-2
			0 - 2	3	4	5	6	7	8			
1		20	4		6	4	3	3		4.15	2.36	20
2		17	6		4	2	1	2	2	3.64	3.06	30
3	13	20	8	2	2	8				2.70	2.41	40
4		20	8		6	6				2.90	2.13	40
5		20	17	2			1			1.30	2.00	80
6		18	9	2	2	4			1	2.33	2.61	50
7	15	18	7	4	5	2				2.33	2.00	38.8
8		20	10		4		2			2.78	2.11	50
9		20	4	2	10	2	2			3.67	1.73	20
10		20	12		4	4				2.22	2.28	60
11	18	20	12	2	2	4				1.89	2.32	60
12		20	6		6	2	6			3.67	2.45	30
13		20	20							0.00	0.00	100
14		20	6		6		4		4	3.78	3.23	30
15		20	12	2	6					1.67	2.00	60
16	20	18	3	1	5	5	4			4.00	2.02	16.6
17		19	1	2	5	4	4	2	1	4.84	1.80	5.26
18	26	20		2	1	2	1	5	9	6.65	1.72	0.0
19		18				1	2	3	12	7.44	0.92	0.0
20	39	18	3	2	6	6	1			3.66	1.84	16.6
21		20	4	5	6	4	1			3.25	1.86	20
22	47	18	1		3	1	2	3	8	6.33	2.19	5.5
23		17	2	3	1	1		4	6	5.52	2.82	11.7

SD: standard division. TRN: titre reference number.

Table (2): Fife week's intervals monitoring of AI HI antibody titres in vaccinated breeder flocks.

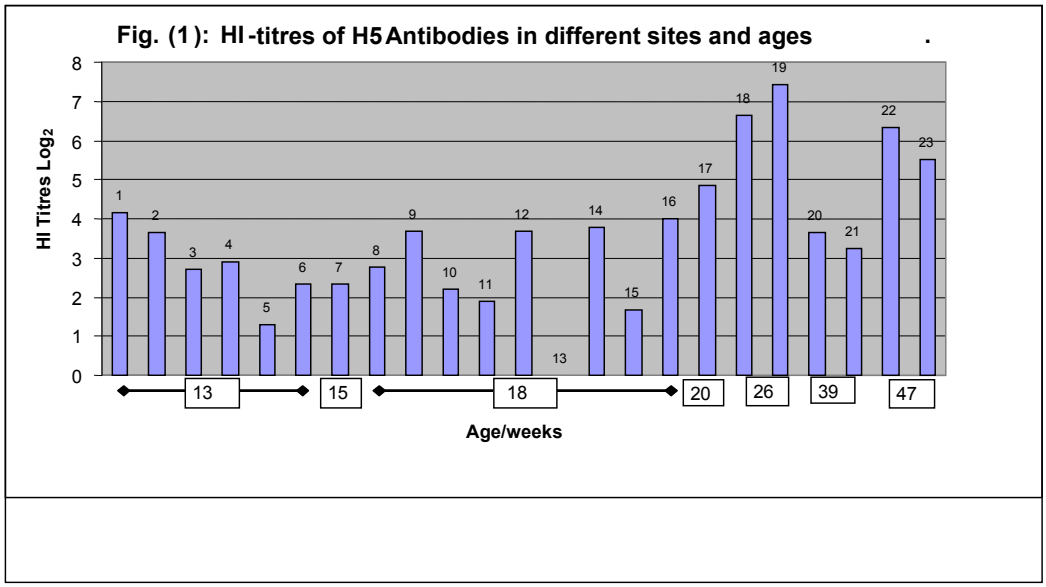
Flock No	Age/ weeks	Distribution of HI TRN - titre							Mean ± SD	
		0-2	3	4	5	6	7	8		
	5	2	2	4	4	1	1	6	5.15	2.52
	10	2	3	4	4	4	1	2	4.60	2.16
	15	4	2	5	6	2		1	3.50	2.24
	20	1	5	6	7	1			4.00	1.29
	25			1	2	6	5	6	6.56	1.18
1	30			1	3	6	5	5	6.50	1.19
	35			3	4	5	3	5	6.2	1.43
	40	1	4	7	3	2	2	1	4.45	1.79
	45			2	5	4	4	5	6.25	1.36
	50		2	3	8	4	2	1	5.20	1.28
	25					4	6	10	7.30	0.81
	30			2	1	4	9	4	6.60	1.18
	35		2	2	4	8	2		5.50	1.23
2	40				6	10	3	1	4.95	0.83
	45	2	2	8	4	4			4.10	1.68
	50				1	7	5	7	6.9	0.96
	55		4	8	2	3	2	1	4.7	1.49

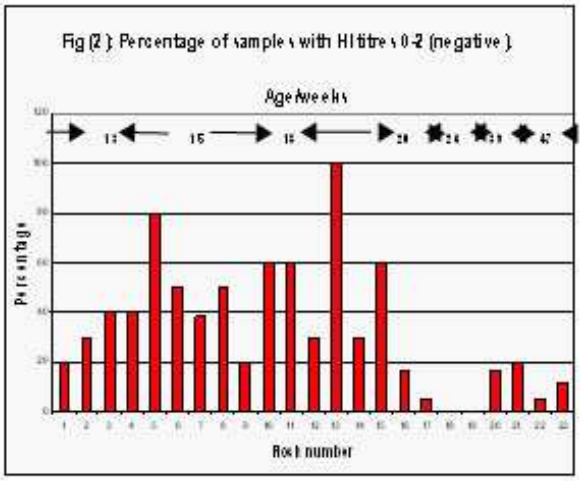
SD: standard division. TRN: titre reference number.

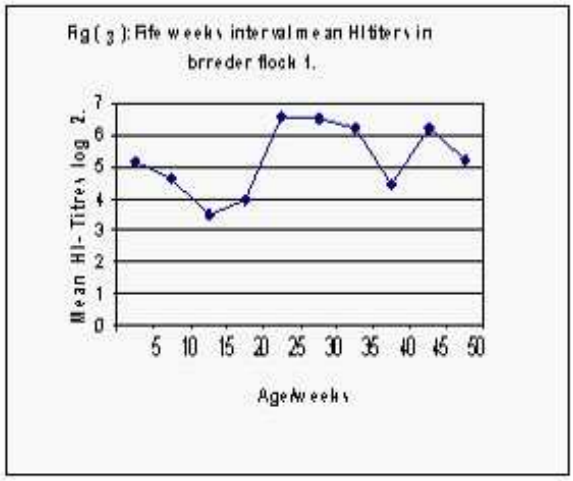
Table (3): Results of HI titre in Female samples compared with males of breeder flocks aged 41 weeks.

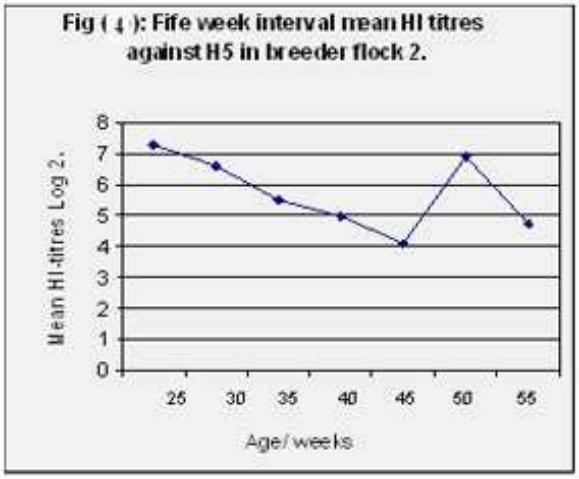
Flock No	Sex	No of samples	Distribution of HI - TRN							Mean ± SD		Flock mean
			0- 2	3	4	5	6	7	8			
1	Female	12			1				11	7.67	1.21	7.73
	Male	3							3	8.00	0.00	
2	Female	10						1	9	7.90	0.33	7.93
	Male	4							4	8.00	0.00	
3	Female	12				1		2	9	7.58	0.93	7.31
	Male	4		1			2	1		5.50	1.73	
4	Female	12							12	8.00	0.00	7.76
	Male	5					2		3	7.20	1.15	
5	Female	12			1			1	10	7.58	1.21	7.57
	Male	2							2	8.00	0.00	
6	Female	12					3	2	7	7.17	0.94	7.46
	Male	3							3	8.00	0.00	
7	Female	12	1	1			1	9		6.17	1.81	6.06
	Male	3				1	1		1	6.33	1.53	
8	Female	12		1	1	1	3	6		6.00	1.38	6.35
	Male	5				1		1	3	7.20	1.30	

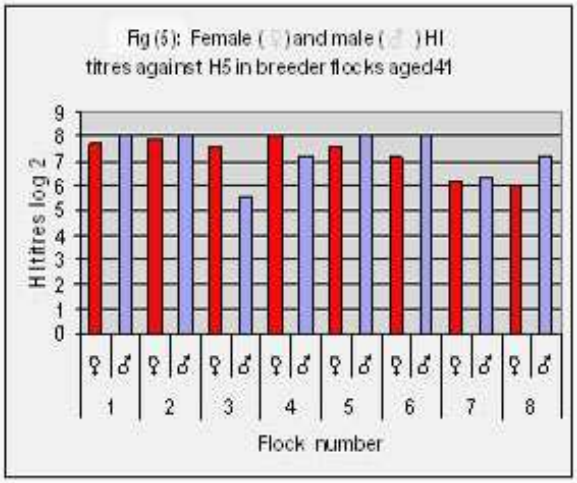
SD: standard division. TRN: titre reference number











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الاستجابة المناعية لأمهات التسمين للقاح أنفلونزا الطيور الميت الزيتي H5N1 تحت الظروف الحقلية

أجريت هذه الدراسة على عينات من أمصال قطعان أمهات التسمين التجاري والمحصنة ضد أنفلونزا الطيور اللقاح الزيتي الميت H5N1شملت هذه الدراسة ٢٣ قطيع تتراوح أعمارها بين ١٣-٤٧ أسبوعا في بيوت مغلقة و موزعة على ٧ مواقع و كذا قطيعين للاستبيان المناعي كل ٥ أسابيع و ٨ قطعان عمر ٤١ أسبوع من مواقع مختلفة وعرفت عينات الدجاجات والديوك المجمعة منها. تم التعرف على الأجسام المناعية لفيروس الأنفلونزا المانعة للتلازن باختبار مانع تلازن الدم HI باستخدام مستضاد فيروسي مماثل للقاح. تلقت القطعان جرعات اللقاح بالحقن تحت جلد الرقبة بجرعة ٠,٢ ملل في اليوم الأول من العمر ثم ٠,٥ ملل عند ١٨يوم و ١٩-٢٠ أسبوع و ٤٠ أسبوع من العمر. أوضح الاختبار المصلى إن الاستخدام الحقلي للقاح الأنفلونزا الميت المحضر من الفيروس عترة H5N1 قد نتج عن الجرعتين الأول تفاوت في مستويات المناعة بين الصقعان والتي تراوحت بين الصفر- ٤,١٥ وكذا داخل القطيع حيث كانت نسب العينات ذات المستوى ٠-٢ بين ٢٠-١٠٠ %. القطعان التي تلقت القرعة الثالثة والتي كان لها الأثر في رفع المناعة إلى مستوى ٣,٢٥-٧,٤٤ مع تجانس العينات داخل القطيع حيث قلت العينات السالبة إلى ٠-٢٠ %. كان لجرعة اللقاح الرابعة عند ٤٠ أسبوع الأثر في تحسين المناعة إلى ٥,٥٢-٦,٣٣ خاصة في مواجهة إجهاد إنتاج البيض. أوضحت نتائج الاستبيان المناعي لعينات جمعت كل ٥ أسابيع لكل من القطيعين أهمية ذلك في تحديد مواعيد وأهمية الجرعات التنشيطية مع عدم تماثل المنحنى المناعي لكل منهما. اختبار عينات الديوك والدجاجات من نفس القطيع أوضح فروق بين٠,٣٣-١,٢ ولكن لم يؤثر هذا الفرق فى المتوسط العام للقطيع. نقل الدجاج السالب فى الاختبار والبالغ من العمر ٤١ أسبوعا للعدوى بالمخالطة لمدة ١٢ يوم وعدم ظهور أعراض المرض أو تغير المستوى المناعى الدموى ليوضح الطبيعة الخاصة لمقاومة الطيور المحصنة للعدوى. أثبتت الدراسة ان استخدام اللقاح الميت المحضر من عترة الفيروس أمماثلة الى حد كبير مع العترة الحقلية فى أربع جرعات كان له الأثر فى إحداث مناعة جيدة والحماية من العدوى بالمخالطة.