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Some studies on Avian Influenza in Egypt
II. Screening on Avian Influenza infection in some chicken flocks

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SUMMARY

During the period 2006 to 2009, a total of 1096 blood samples from 92 chicken flocks (21 of breeder flocks,63 of commercial layer flocks and 8 broiler flocks), were collected from different governorates (Giza, Qalubia, Sharkia and Dakahlia) and tested for determination of hemagglutination-inhibition (HI) titers against Avian Influenza (AI). All of the surveyed farms applied blind vaccination programs without serological estimation to MDA and actively acquired humoral immune response to determine timing of priming or boosting(s). HI titer equal to or less than 2^4 was detected in 76 flocks (82.6%), and there were 16 flocks (17.4%) showed low antibody titer (<4 log2). The results indicated that MDA persisted for 28 days duration after hatchling and these MDA may interfere with early vaccination (less than 2 weeks of age). Early vaccinations within first week of age, with full vaccine dose, were frequently and blindly applied due to panic of AI. The results of evaluation of such flocks at marketing age (30 - 42 days) revealed suboptimal HI titer.

For the control of avian influenza, a rapid diagnosis by detecting the causative virus and identifying its subtype is essential. A rapid diagnosis kit for identification of AI by rapid antigen kits (Type A and H5 kit) was used for detection of AIV in three hundred samples, positive samples were 67, (22.33%). Out of 82 samples from Qalubia, positive samples were 18, (21.95%), samples from Giza were 56, positive samples 11 were positive, (19.64%), 13/68 samples from Dakahlia were positive (19.11%) and 25/94 samples from Sharkia were positive (26.59%).

INTRODUCTION

Influenza A viruses can be classified into various subtypes on the basis of antigenic differences between the two surface

glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Serologically, 16 subtypes of HA (H1–H16) and 9 subtypes of NA (N1–N9) have been identified (Fouchier et al., 2005).

Influenza A viruses are responsible for major disease problems in birds, as well as in mammals including humans. Infection of domestic poultry by AI viruses typically produces syndromes ranging from mild, localized infection such as respiratory disease and drop in egg production to severe, systemic disease with near 100% mortality. Disease is usually absent with AI virus infection in most wild aquatic bird species, which is the primordial reservoir of all influenza A viruses (Swayne and Halvorson, 2003). Highly pathogenic avian influenza (HPAI) can cause severe losses to poultry industries and posses a threat to public health (Capua and Marangon, 2006).

HPAI virus H5N1 was emerged in Egypt in Mid-February 2006 and the disease affected all poultry production sectors causing sever socio-economic losses (Aly et al., 2006-a and b).

For the control of avian influenza, a rapid diagnosis by detecting the causative virus and identifying its subtype is essential. A rapid diagnosis kit combining immunochromatography with enzyme immunoassay which detects the H5 HA antigen of influenza A virus was developed using newly established anti-H5 HA monoclonal antibodies. (Tsuda et al., 2007).

The aim of the present study was planned for virological and serological surveillance on vaccinated chicken flocks with AI vaccines.

MATERIAL AND METHODS

1. Tissue samples and swabs:

Three hundred samples (tracheal, cloacal swabs and tissues either sinuses or trachea or lungs) were collected from four governorates (Giza, Qalubia, Sharkia and Dakahlia) from commercial flocks, backyards and live bird markets. Examined flocks were sufferd from septicaemia and signs as high mortality, respiratory signs, nervous signs, and drop in egg production with septicemic pictures. The samples were collected from live and/or freshly dead birds. The samples were placed in 1-2 ml sterile isotonic phosphate buffered saline (PBS) containing penicillin and streptomycin as transport media, pH 7.0-7.4.

2. Serum samples:

One thousand and ninety-six blood samples for sera were collected from different vaccinated flocks. A total of 92 flocks were tested the titer of antibodies against H5 in vaccinated chickens (21 flocks from breeders, 63 flocks from commercial layers and 8 flocks from broilers), collected from four governorates (Giza, Qalubia, Sharkia and Dakahlia). Serum samples were carefully

separated in small vials and kept at – 20 °C till used.

3. Reference Antigens and antisera for HI:

a)Three types of AIV hemagglutinating antigens (one H5N1 and Two H5N2) which represented the homologus and hetrologus antigens of the follow mentioned vaccines and were obtained from obtained from local agency and were used in HI test.

b) Known positive and negative AIV antisera were obtained from GD, Holland Marketing International Center, obtained from local agency, and were used in HI test.

Hemagglutination and hemagglutination inhibition (HI) tests:

The recommended method use V-bottomed micro well plastic plates was applied. In which the final volume for both types of HA and HI test was 0.075 ml. The reagents required for these tests are isotonic PBS (0.1 M), pH 7.0-7.2 and RBCs. Positive and negative control antigens and antisera should be run with each test. HI titers may be regarded as being positive if there is inhibition at a serum dilution of 1:16 (2^4 or 4 log-2 when expressed as the reciprocal) or more against 4 HAU of antigen according to OIE manual (2005).

kits used for AI antigen detection:

a. Group antigen-strip type A AI: Commercial kit Influenza type A antigen test kit. Anigen, Animal Genetics inc. Soluo, Korea.

b. Antigen-strip H5 Sub-type: Commercial kit Antigen Rapid H5 Avian influenza Virus Antigen Test Kit (Antigen H5 Rapid Test Kit). Anigen, Animal Genetics inc. Soluo, Korea.

RESULTS AND DISCUSSION

For the control of avian influenza, a rapid diagnosis by detecting the causative virus and identifying its subtype is essential. Strip type a influenza or H5 test kits is more rapid method need only 20 minutes to preliminary diagnosis of the positive cases especially in high titters more than 10^2, but the negative results must be confirmed by PCR. The kit specifically detected all of the H5 influenza viruses tested, and did not react with the other HA subtypes. H5 HA antigens were detected from swabs and tissue homogenates of chickens infected with HPAI virus strain begging from 2 days post infection. The kit showed enough sensitivity and specificity for the rapid diagnosis of HPAI (Tsuda et al., 2007). The kit use a monoclonal Ab against the nucleoprotein and able to detect any influenza A viruses. Although it was developed to detect virus in mammalian infection, it has been successfully applied to detecting viruses in poultry and other birds, although they may some variation in the sensitivity for different specimens (OIE Manual 2005).

In our study, the clinical data of the studied outbreaks were highly suggestive of being due to HPAI or velogenic ND viruses. The examined birds suffered from sings of septicemia and hemorrhages on the shanks of the infected chickens. On necropsy, the dead birds showed congestion of internal organs, hemorrhages on the coronary fat, pancreas, ovary and brain (Capua et al., 2002). Three hundred cloacal and tracheal swabs were subjected for antigen rapid kit type (A) firstly, and then the positive samples were examined by antigen rapid kit H5. Eighty two samples from Qalubia, 56 samples from Giza, 64 samples from Dakahlia and 98 samples from Sharkia (table 1). Some of these samples were taken from flocks have high HI antibodies titer and gave the positive result for H5 antigen rapid kit (Fig 1), although this flock vaccinated 3 times with H5N2, while newborn flock have a moderate geometric mean HI titer 7, and gave the negative result with H5 rapid kit. This may be the virus can't be detected by this kit or no virus exposed to this flock. These results indicated that, the vaccine used in the field is not enough for the control of AIV, whereas the virus can escape and induce a disease in the flocks especially vaccinated with some vaccines of low quality (H5N2, or even H5N1). Some flocks gave negative results of H5 rapid kit and have a high titer of HI antibodies (10.3 geometric mean), These explain that, neutralizing N1 antibodies may play a role in the protection and shedding of H5N1 AI virus. Safwat (2006) recorded the first isolation of AI H5N1 in Egypt during the period of 12/2/2006 to 14/2/2006 in 3 provinces (Cairo, Kaliobia and Giza). The virus isolated from backyard duck and geese from freshly dead carcasses. The AI isolation was confirmed by influenza type A antigen test strip kit as a rapid detection methods for avian influenza_type A antigen. Fuhu Peng et al., (2008) recorded the advantages of rapid detection and easy operation without the requirement for special skills and equipment makes the strip suitable for onsite detection and the differentiation of H9AIVs from other viruses in poultry. Also, Loth et al., (2008) reported that, these tests are a valuable tool for the Indonesian avian influenza control program by reliably and quickly detecting Influenza A virus from oropharyngeal swabs from sick or dying chickens.

Table (1) Results of the Antigen Rapid Kit AI type (A) & subtype (H5):

Site of Samples(Governorate)	Number of Examined Samples	Positive Results of Antigen Rapid Kit	% of Positive
Qalubia	82	18	21.95%
Giza	56	11	19.64%
Dakahlia	68	13	19.11%
Sharkia	94	25	26.59%
Total	300	67	22.33%

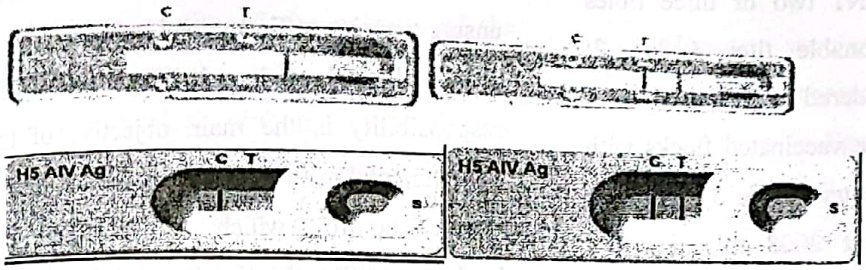

Fig. (1): Negative and positive samples by rapid antigen kit H5

Estimation of post vaccinal antibody titer is a routine laboratory process to ensure the efficacy of the vaccination program. HI is the test of choice for estimation of antibody titer for AI vaccination (OIE Manual, 2005). our studies deal some serological survey levels on surveyed different poultry farms and provide the level of humoral immunity measured by (HI-test) in vaccinated poultry flocks within surveyed different chicken farms (breeders, commercial layers & broilers), among different governorates as a part of whole AI control measures (Tables 2, A,B and C) (USAID, 2007).

A total of 92 chicken flocks (1096 blood samples) were tested for detection of hemagglutinating antibodies against AIV in vaccinated chickens (21 of breeders , 63 of commercial layers and 8 broiler flocks),collected from four governorates (Giza, Qalubia, Sharkia and Dakahlia). Hemagglutination-inhibition (HI) titers were determined according to standard methods (OIEManual, 2005). An HI titer more than 2^4 or 4 log-2 was detected in 76 flocks (82.6%), and there were 16 flocks (17.4%) showed low antibody titer (<4 log2) table (3). Similar data

reported by Aly et al., (2007), El-Samadony (2008) and Hussein et. al. (2008).

The examined vaccinated flocks have variable hemagglutinating antibodies titer according to the type of vaccine used either H5N1 or H5N2 even different vaccines of H5N2 whereas there is difference in the seed virus strain used in the vaccine. The similarity of the profile of H5 used in the vaccine play a major role in the protective titer. Some flocks vaccinated with H5N1 two or three times have a high reasonable titer 4 log 2; previously was considered protective (Tian et al., 2005) than some vaccinated flocks with H5N2, the same boosters (table 3) that have lower titer (while at 2008 this titer not protective unless the titer above 7 log 2 (Swayne, 2008 and Hussein et. al. 2008). Besides, on field observation, these vaccinated flocks can overcome the field challenge. On the other hand, some vaccinated flocks with H5N2 showed high HI titer although the vaccination was two times compared to flocks vaccinated with H5N1 three times. In the same time, some of these flocks (vaccinated with H5N2) exposed to HPAI outbreak with high mortality (45%), on historical aspects of these flocks declared that, these flocks infected with IBD outbreak at young ages (Afify, personnel communication).

Concerning the serological survey the main following critical observations were reported:

1-It is worthy noting that the general biosecurity rules for poultry production (Borne et al., 2007) and those specifically for AI control (Shapiro and Brown, 2008).

2-Surveyed poultry farms sold their end product at the end of crop cycles as live birds, which reflect the sever lack of slaughter houses, thus contribute in the spread of the AI virus and allows endemic of AI.

3-Quality control of AI-vaccines which ensure vaccine efficacy and potency based principally judged by challenge test. Such responsibility is the main objective of the governmental central laboratory for veterinary Quality control, which is lacked at the beginning of outbreak due to absence of biosafety lab- 3 (BSL) level, which consequently reflected on presence of more than 17 inactivated AI vaccine in the currently available in the local market, and some of them are fake vaccines, which well be emphasized in the present investigations However, in 2009, they applied challenge test.

4-The majority of the surveyed farms applied blind vaccination programs without estimation of the MDA and level of actively acquired immune response to determine time of boosting (s).

These findings suggest contribution of several explanations including:

(1)The roles of presence of MDA at vaccination, where it interfere with vaccine

take (Swayne, 2006-a. Gardin, 2007 and Sultan and Hussein, 2008).

(2)Vaccination at early age (less than 2 weeks of age) of non immune competent age, where basis of priming vaccination must be at least at 2 weeks age to ensure optimum immune response (Swayne and Kapczynski, 2008); or at about 3 weeks of age to be fully efficacious (Stone, 1987);

(3)Differences in obtained HI- titer levels resulted with Al- vaccines, suggest variation in quality of the potency of the applied vaccines.

(4)Individual bird not responded to vaccination at the end of fattening period and at marketing age, can suggest the importance of the issue of field virus laboratory protection, where filed protection is less than achievable in the laboratory because, of immunosuppressive viruses, vaccine storage and transport problems, in complete or missed vaccination of poultry on a farm or within a region, and failure to follow manufacturer label including usage of reduced vaccine dose administration (Swayne, 2003 and Swayne, 2004).

This data strongly emphasized the presence of suboptimal vaccine quality in the local market, which the importance of presence of high biocontaminent facilities of BSL-3 for titration and quality control of such vaccines Ministry of agriculture and authorized organization (GOVS), must in force- establishment of such laboratory in veterinary serum and vaccine research institute (VSVRI) to assess protection and consistency of vaccine batches as means to ensure a minimal protective level (Maas et al., 2000).

However, vaccination will continue to be used as a key component in the control of avian influenza in Egypt as many countries like China (including Hong Kong SAR), Viet Nam, Indonesia and Russia. Vaccination reduces the number of susceptible poultry, raises resistance to infection, and reduces the amount of virus that immune infected poultry excrete. Antigens in adjuvanted poultry vaccines do not have to be a perfect match to provide protection; HA antigens in vaccines should ideally be a close match to field strains and sufficient antigen included to ensure strong immunity (Swayne 2003).

Table (2, A) Serological response of vaccinated breeder flocks with inactivated AI-vaccines as measured by HI test (using 4 HA unit).

Flock No.	Age/ days	Sex	No. of Ex. Sera	HI-titers Log.2													MT
				0	1	2	3	4	5	6	7	8	9	10	11	≥12	
1	150	F	16				1	1	1	3	1	3		3	1	2	7.9
2	150	M	4				1			1	1					1	7.0
3	150	F	16						1	1	7	1	4	2			7.8
4	150	M	4						2	1	1						5.8
5	224	F	13							1	1	5	3	2	1		8.5
6	224	M	2								1	1					7.5
7	44	F	16	1		5	3	3	3	1							3.2
8	44	M	4		1		1	1	1								5.1
9	60		15		1		1	2	3	6	2						5.1
10	60		15		1			5	1	6	1		1				5.2
11	30		20			1			1	4	9	3	1			1	7
12	30		20	1			2		2	8	4	2	1				5.9
13	65		20				2	3	4	7	3	1					5.5
14	65		20				4	3	4	5	3	1					5.2
15	30		20									1	3	4	7	5	10.6
16	30		20				1	1	3	3	3	4	3	2			7.0
17	65		24							1	1		3	7	8	4	10.3
18	55		20						1	2	10	3	3	1			7.4
19	55		20					1		5	3	9	2				7.3
20	36		10							1	1	2	3	3			8.6
21	36		10	1	1	2	2	2	2								2.9

Table (2, B) Serological response of vaccinated commercial layer flocks with inactivated Al-vaccines as measured by HI test (using 4 HA unit).

Flock No.	Age / Days	No. of Ex. Sera	HI- titers Log. 2													MT
			0	1	2	3	4	5	6	7	8	9	10	11	≥12	
1	35	20	1		4	2	5	1	5	1	1					4.2
2	77	9	1	2	2	1	2	1								2.4
3	77	11		1		4	4	2								3.5
4	92	14							1	2	5	3	3			8.3
5	92	14						1	1	3	8	1				7.5
6	92	14									3	5	5	1		9.3
7	37	18							2	6	4	4	2			7.9
8	37	20						2	1	4	2	7	1	2	1	8.4
9	35	21								2	1 1	5	3			8.4
10	12	13								6	5	1	1			7.8
11	40	23								2	6	10		4	1	8.8
12	16	10						2		2	1		4	1		8.3
13	16	10							1	2	4	3				6.9
14	10	21			6	8	5	2								3.1
15	10	13			6	2	3	1	1							3.2
16	10	23			2	7	6	6	2							4.0
17	20	15						5	5	2	2	1				6.2
18	20	15			3	3	4	1	2	2						3.3
19	20	10						1	2	4	2	1				7.0
20	36	25						1	5	6	4	7	2			7.7
21	33	25					1	2	5	8	7	2				7.0
22	26	25	1	2	1		3	5	4	3	5	1				5.4
23	23	25			1		5	8	6	3	1	1				5.4
24	22	10						2	3	1		2	2			7.3
25	22	6					1	3		1			1			6.0
26	22	6					1	2		1	1	1				6.3
27	22	10						1	1	2	5		1			7.5

Continue Table (2,b)

Flock No.	Age / Days	No. of Ex. Sera	HI- titers Log. 2													MT
			0	1	2	3	4	5	6	7	8	9	10	11	≥12	
28	22	6			1			1	1	1	1		1			6.3
29	22	6				1			5							5.5
30	65	10								1		4	4	1		9.4
31	65	10									2	3	3	2		9.5
32	65	10					1	1	3	4	1					6.3
33	65	10			1			1	5	1	1	1				6.1
34	65	10								3	4	3				8.0
35	65	10							1	2	4	2	1			8.0
36	40	9		2	3			1	1	1	1					3.8
37	40	9		2		1	2		2	2						4.3
38	40	9				1	3	4			1					4.8
39	40	6		1	1	2			1	1						3.7
40	40	9	1	1	1			3	3							4.0
41	40	9	3	2	3		1									1.3
42	40	9	1	1	2	1	1	1	1			1				3.4
43	35	5						3	2							5.4
44	35	5							1	2	2					7.2
45	35	5							1	1	3					7.4
46	35	5					2			3						5.8
47	35	5						1	3		1					6.2
48	35	5						2	1	1	1					6.2
49	35	5						3	1				1			6.2
50	35	5											5			10.0
51	35	5											5			10.0
52	35	5						2	1	2						6.0
53	35	5			1		1	1	1	1						4.8
54	25	24						1	2	5	1	7	4	4		8.6
55	25	26							1	1	6	3	3	12		9.6
56	25	25					1	1	1	1	2	3	3	7	6	9.8
57	25	25						5	4	9	4	2	1			6.9
58	25	25					1	2	1	1	1	2	4	7	6	9.7

Continue Table (2,b)

Flock No.	Age / Days	No. of Ex. Sera	HI- titers Log. 2													MT
			0	1	2	3	4	5	6	7	8	9	10	11	≥12	
59	25	25							3	11	5	2	4			7.7
60	45	7							1	1	3	2				7.9
61	45	7						1	1	1	3	1				7.3
62	55	20					1	1	1	6	10		1			7.4
63	55	20								4	4	7	2	3		8.8

Table (2, C) Serological response of vaccinated Broiler flocks with inactivated AI-vaccines as measured by HI test (using 4 HA unit).

Flock No.	Age / Days	No. of Ex. Sera	HI- titers Log. 2													MT
			0	1	2	3	4	5	6	7	8	9	10	11	≥12	
1	28	10									2	7	1			8.9
2	28	10								2	5	1	2			8.3
3	34	10	1		1	2	4		2							3.6
4	34	10	3	1	3	1		2								2.0
5	40	10						2	4	2	2					6.4
6	40	9						5		2	1	1				6.2
7	40	10	2		1	3	3	1								2.8
8	40	9				2	4	2	1							4.2

It is known that the immune response produced by a dose of antigen that will prevent disease signs that required to reduce viral shedding to undetectable levels. This indicates that the amount of antibodies in the blood stream may effectively prevent the systemic or viremic phase of disease caused by HPAI viruses and may partially explain the broad protection against HPAI challenge, it seems clear that the efficacy of the vaccine depends primarily on the dose and antigenic relatedness of the circulating viruses with the strains used for vaccination.

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بعض الدراسات عن انفلونزا الطيور فى مصر
2. استقصاء فيرولوجى و سيرولوجى لفيروس أنفلونزا الطيور فى بعض قطعان الدجاج

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تم فحص عدد 1096 عينة سيرم تم جمعهم من 92 قطيع للطيور (21 قطيعا للأمهات و63 قطيعا للبياض 8 قطعان للتسمين) تم جمعهم من محافظات الجيزة – القليوبية – الشرقية – الدقهلية. للكشف عن الأجسام المضادة لمرض أنفلونزا الطيور للنوع الفرعي (H5) وذلك بعد التحصين بلقاح أنفلونزا الطيور المخمد (الغير نشط) وذلك خلال الفترة من 2006 إلى 2009.

حيث تم فحصهم معمليا باختبار مانع التلازن الدموي لتحديد منسوب الأجسام المناعية الناتجة بعد التحصين تم اكتشاف منسوب الأجسام المناعية المانعة للتلازن الدموي بمعدل أعلى من 4 لوغاريتم 2 و ضد الإصابة بمرض أنفلونزا الطيور في عدد 76 قطيع بنسبة 82.6% و عدد 16 قطيع بنسبة 17.4% كان فيهم منسوب الأجسام المناعية المانعة للتلازن الدموي بمعدل أقل من 4 لوغاريتم 2 ضد الإصابة بمرض أنفلونزا الطيور. التحصين المبكر في خلال الأسبوع الأول من عمر الكتاكيت و بجرعة تحصين كاملة و ذلك بسبب الذعر من مرض أنفلونزا الطيور حيث دلت النتائج أن هذه القطعان في أعمار التسويق (30-42 يوم) لم يكن بها مستوى مناعي و حماية كافية ضد المرض.

للسيطرة على مرض أنفلونزا الطيور كان من الضروري عمل اختبار التشخيص السريع للكشف عن الفيروس المسبب للمرض و تحديد فرعيته حيث تم عمل اختبار الكشف السريع للأنتيجين على عدد 300 عينة (مسحة قصبة هوائية و مجمع) من محافظات القليوبية – الجيزة – الدقهلية – الشرقية حيث كان منهم عدد 67 عينة ايجابية بنسبة 22.33% وكان توزيعهم كالتالى: عدد 82 عينة من محافظة القليوبية حيث كان منهم عدد 18 عينة ايجابية بنسبة 21.95% ، عدد 56 عينة من محافظة الجيزة حيث كان منهم عدد 11 عينة ايجابية بنسبة 19.64%، عدد 68 عينة من محافظة الدقهلية حيث كان منهم عدد 13 عينة ايجابية بنسبة 19.11%، عدد 94 عينة من محافظة الشرقية حيث كان منهم عدد 25 عينة ايجابية بنسبة 26.59%، و تم تأكيد النتائج الايجابية بعمل اختبار التفاعل الأنزيمي المتسلسل في الوقت الحقيقي. تم إجراء الاختبار كاختبار مؤكد لتأكيد كشف فيروس أنفلونزا الطيور في العينات المفحوصة لمقارنته باختبار التشخيص السريع.