Comparative Studies On The Immune Response Elicited In Chickens Vaccinated With Recombinant Avian Influenza-Fowl Pox Vaccines As A Primary Vaccine Or Prime-Boost Scheme

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ABSTRACT

Comparative immunological studies on different types of Live recombinant Fowl Pox virus containing the avian influenza H5 hemagglutinin (HA) gene (rFP-AI-H5) from several strains were done to evaluate their efficacies. Susceptible SPF chickens were vaccinated with the rFP-AI-H5/Scotland and rFP-AI-H5/Irland vaccines as primary or prime-boost vaccination with killed H5N2 vaccine to study the immune response against them through monitoring protection % against local circulating highly pathogenic avian influenza (HPAI) H5N1 strain 28 days post-vaccination. All vaccinated chickens with one or double doses of live recombinant vaccines and challenged with HPAIV succumbed to disease, while those vaccinated with killed vaccine or recombinant vaccines as prime-boosted by killed AI vaccine were protected from severe clinical signs and death. Both rFP-AI-H5/Scotland and rFP-AI-H5/Irland vaccines induced poor protection % when used as one dose (17.7% and 37.5%) or as two doses (23.5% and 47.1%) against HPAIV. In birds vaccinated with the inactivated AI-H5N2 as primary (either one or double doses) or boosting to the recombinant vaccines (rFP-AI-H5/Scotland and rFP-AI-H5/Irland) became protected after challenge by HPAIV with 87.5, 100, 88.2, 94.1%. By using oropharyngeal swabs from the live infected control group as well as the vaccinated chicken, it was observed that the reduction in the viral shedding were 0.3, 0.7, 0.5, 1.3, 2.4, 3.9, 2.9 and 3.2 corresponding for chicken vaccinated with one and two doses of rFP-AI-H5/Scotland, one and two doses of rFP-AI-H5/Irland one and two doses of inactivated H5N2 and rFP-AI-H5 (Scotland and Irland) boosted with inactivated H5N2, respectively. The data clearly indicate that the inactivated AI vaccine confers protection comparable to that of the recombinant as primary vaccine against AIV. While, rFP-AI-H5/Irland was more effective than rFP-AI-H5/Scotland.

INTRODUCTION

The control of avian influenza (AI) depends on eradication strategies in some countries but this policy had led to very high cost and economical losses. Other countries depend on the vaccination strategies especially in areas with high animal densities leading to increased risk of disease spread *(1)*. Vaccination against AI has proven to be a successful additional control measure implemented along side controlled culling *(2)*.

Inactivated, whole virus vaccines were considered the main type that are licensed widely by several countries and have proven efficacy.

Other types of live virus vaccines have been developed for AI using alternative recombinant live vectored constructs and can provide some of the immunological advantages of live vaccines but without the reassortant risk of live AI virus *(3)*. Moreover, the disadvantages of some live recombinant vaccines include the risk of generating revertants and allow spread of genetically modified organisms in the environment *(4)*.

These vaccines use recombinant DNA technology to incorporate genetic material provided from the AI genome into a viral backbone for gene expression in vivo where the vector acts as a carrier and may itself act as a protective immunogen. Many examples of these types of vaccines have been documented with varying levels of success as Fowl pox with H5 (rFP-AI-H5) *(5)* and Lasota strain Newcastle with H5 (rND-AI-H5) *(6)*, some of which have been reported to be efficiently protect chickens against HPAI *(7)*.

So, this study was aimed to compare the immune response of the different types of rFP-AI-H5 vaccines as primary or prime-boost vaccination with killed H5N2 vaccine through monitoring protection % against local circulating highly pathogenic avian influenza (HPAI) H5N1 strain.

MATERIAL AND METHODS

1. Laboratory animals

a) Embryonated chicken eggs

A total of 70 specific pathogen free SPF Embryonated Chicken Eggs (ECEs), 12-13 day old, were used for performing titration of live vector vaccine, preparation of challenge virus and viral shedding. These eggs were obtained from Kom Oshim farm for SPF-eggs, El-Fayoum, Egypt.

b) Experimental birds

A total of 220 SPF chickens were obtained from Kom Oshiem, El-Fayoum Farm as one day old. They were maintained at Central Laboratory for Evaluation of Veterinary biologics, Abbasia, Cairo (CLEVB) and housed in positive pressure stainless steel isolation cabinets with continuous light exposure till used.

2. Vaccines

a) Live Recombinant Fowl Pox-AI vaccine (rFP-AI-H5)

Lyophilized vaccine contains a suspension of a live recombinant Fowl Pox virus used as vector containing an insert of the heamagglutinin subtype H5 gene of avian influenza virus. They were produced by Boehringer Inglhiem vetmedica S.A. de .C.V. Guadalajara, jal. Mexico and Merial, france where the H5 gene of AI is derived from the vaccinal strains A\ Chicken\ Scotland\59 (rFP-AI-H5/Scotland) and A\Chicken\ Irland\83 (rFP-AI-H5/Irland), respectively. The vaccine was administrated in one day old chicks by subcutanous route in a dose 0.2 ml per bird.

b) The imported inactivated H5N2 AI influenza vaccine

The inactivated oil emulsion LPAI H5N2 vaccine was produced by Boehringer Inglhiem vetmedica S.A. de .C.V. Guadalajara, jal. Mexico. The vaccinal strain is A/Chicken/ Mexico/232/94/CPA. It was administrated to 3 weeks old chickens subcutaneously at the lower third of the neck in a dose of 0.5 ml /bird.

3. Antigens and antisera

The standard H5 antigen and antisera were obtained from GD, Holand and used in identity test for the killed vaccine.

4. Culture media For swabs processing

Tryptose Phosphate broth

Code NO. 0060 - 01- Difco Laboratories, Detriot, Michigan, USA. It was used in cultivation of oropharyngeal and Cloacal swabs for detection of viral shedding.

5. AI challenge virus

Local HPAI field isolate was used as challenge virus. It was isolated and identified by National Laboratory for Quality control of Poultry (NLQP) as A / Chicken / Egypt / 1709-6 / 2008 (H5N1). Its titer was 1010 EID50 / ml. The challenge dose was adjusted to be 105 EID50 / 0.1 ml per bird and administered intra-nasal.

6.Titration of live recombinant vaccine *(8)*

The titer of live vector (rFP-AI) vaccine carried out for the fowl pox virus according to OIE 2008 using SPF 13 day old Embryonated Chicken Egg (ECE) on the chorio-allantoic membranes (CAM). The surviving ECE were examined for evidence of pock lesion, five days post inoculation.

7. Identity test *(8)*

a) In case of inactivated vaccines

The identity of AIV type incorporated in the vaccine under test is carried out through testing of sera collected from vaccinated chickens (in conjunction with potency test) by HI test using standard H5 AI antigens.

b) In case of live vector vaccine

For detection of the H5 gene insert in the recombinant FP-AI vaccines, the PCR procedure was done *(9)*. The viral DNA was extracted using DNA extraction kit (QIAamp DNA mini kit # 51304, Qiagen). Then the PCR was conducted according to PCR instructions (AmpliTaq Gold DNA polymerase kit # N808-0240, Roche) using two pairs of primers specific to the AI inserts.

The PCR reaction scheme was one cycle at 95°C for 5 min, and 30 cycle (94°C for 1min, 58°C forl min, and 72°C for 2min) and one cycle of 68°C for 7 min. The amplified segments were separated on 1 % agarose gel.

8. Potency and effecacy test (8)

SPF chickens, one day and three weeks old, were vaccinated S/C with field dose recommended by the productive companies for recombinant and inactivated vaccines respectively. All the chicken groups were boosted with one field dose of inactivated AI-H5 vaccine, 2 weeks post the first vaccination. Seventeen birds out of each vaccinated and non-vaccinated groups were challenged intranasally by local Egyptian HPAI H5N1 isolates (A \ Chicken \ Egypt \ 1709-6 \ 2008) 4 weeks post the last vaccination. The challenge virus dose was 0.1 ml containing 10^5 EID/ml. All the birds were observed daily for 10 days post challenge (pc). Three days pc, the morbidity and mortality rates were recorded for each group till the end of the observation period to measure the protection %. The assessment of viral shedding due to replication of HPAI challenge virus were performed *(8)* through collection of oropharyngeal swabs in tryptose media containing antibiotic mixture on 2nd days post challenge from the live infected control group as well as the vaccinated chickens using 9-11 day old SPF ECE.

9. Experimental Design

In this study, SPF chickens were used to evaluate the efficacy of different imported live recombinant (rFP-AI-H5 Scotland and Ireland) and inactivated avian influenza vaccines (Mexican H5N2). The birds vaccinated with rFP-AI-H5 vaccines were divided into 2 experimental groups 1 and 2 (75 birds /each), each group was divided into 3 subgroups, one had 25 birds for one dose of each vaccine, the second had 25 birds for two doses and the third had 25 birds for prime-boost of each vaccine with inactivated one. The birds group 3 (50 birds) vaccinated with inactivated AI vaccine were divided into 2 subgroups, one had 25 birds for one dose of inactivated vaccine and the second had 25 birds for two doses.

Control group (4) had 20 birds and sub-divided into 2 subgroups (10 birds /each), the first one represent the infected birds at 4 weeks post 1st vaccination (WP 1st V) and the second subgroup used for challenge at 4 weeks post 2nd vaccination (WP 2nd V) as shown in the following figure:

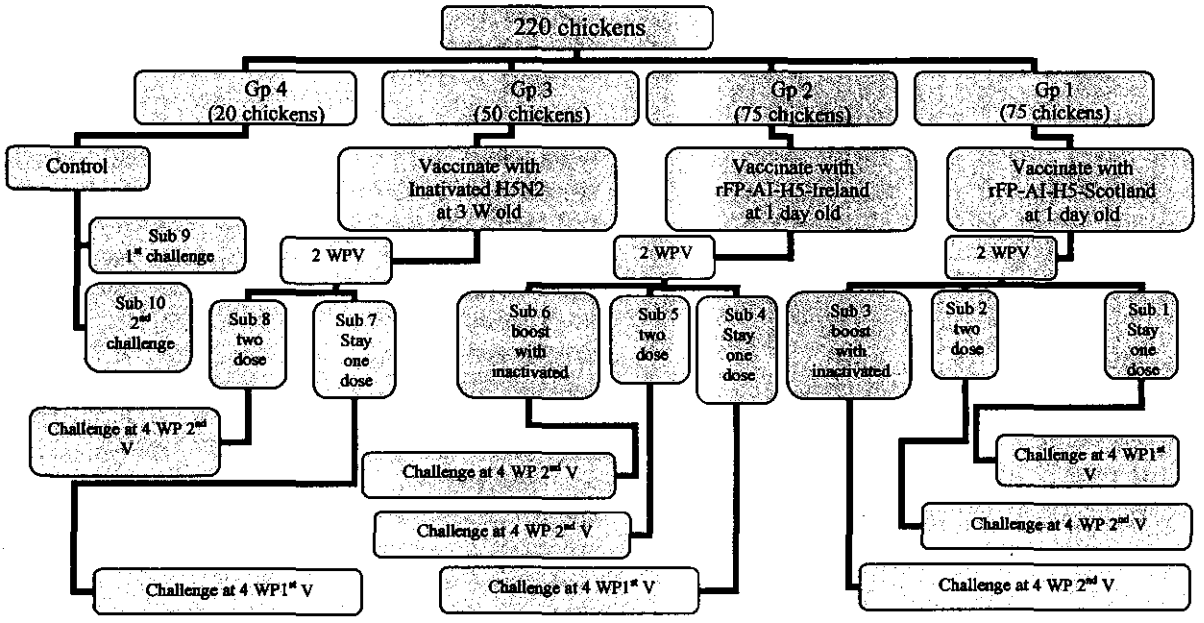

Fig (1): The design of the efficacy experiment for each type of AI vaccines

RESULTS

1.Results of Identity test

a) Inactivated vaccines

It was shown that all the sera collected from vaccinated chickens and tested by HI test using standard homologous H5 AI antigens, gave positive results. It means that the produced HA antibodies were identical to the used antigen and proved that the AI strain contained in the inactivated vaccine was to be H5 subtype.

b) rFP-AI-H5 vaccines

Concerning the rFP-AI-H5 vaccines evaluation results, the most important point were that the recombinant vaccine proved to be containing H5 gene as detected by PCR in the presence of a pair of forward and reverse primers leading to generation of specific PCR fragments which appeared to be of the correct size when analyzed on 1 % agarose gel. Photos (1 and 2) show the amplification of 400bp for rFP-AI-H5/Scotland and 424bp for rFP-AI-H5/Irland which include a region of the HA (H5) of the AI.

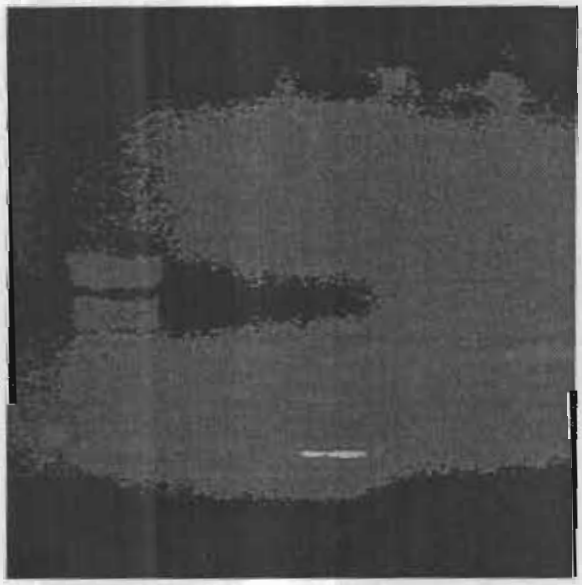

Photo (1)

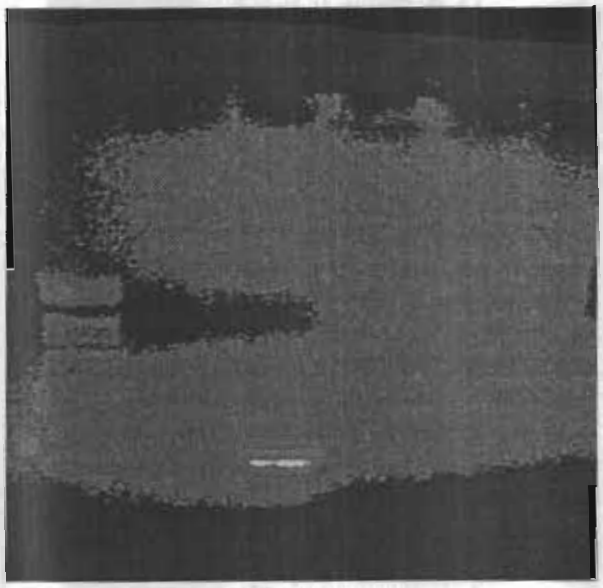

photo (2)

Results of rFP-AI-H5 vaccines Titration test

It was observed that the titer of rFP-AI-H5-Scotland and rFP-AI-H5-Ireland vaccines were $10^{3.6}$ and $10^{3.8}$ EID_{50} / dose when tested in the SPF ECE.

Results of immune response of chickens vaccinated with one dose of different types of AI vaccines

Results of protection% of chickens vaccinated with one field dose of either inactivated H5N2, rFP-AI-H5-Scotland and rFP-AI-H5-Ireland vaccines for avian influenza after challenged by local Egyptian HPAI H5N1 virus are described in table (1). It was observed that in case of imported inactivated H5N2 vaccine, 87.5% from challenged chickens were protected from the disease during I0 days post challenge (DPC). But chickens vaccinated with rFP-AI-H5-Scotland and rFP-AI-H5-Ireland vaccines gave low protection level reach 17.7% and 37.5% respectively. However, the control group that received the same challenge dose, 100% chickens showed death with severe symptoms during 3 days PC.

Also, it showed that there was reduction in local HPAI challenge virus replication either $10^{0.3}$ or $10^{0.7}$ EID_{50} from oropharyngeal swabs in case of chicken groups vaccinated with one dose of rFP-AI-H5-Scotland and rFP-AI-H5-Ireland vaccines. While, it could be deduced that the one dose from inactivated H5N2vaccine evoked a reduction in the challenge virus dose shed from respiratory tract equal $10^{2.4}$ EID_{50}.

Table 1. Results of the efficacy of single dose from different AI vaccines against challenge with local HPAI virus at 4 Week post vaccination

Vaccine type	No. of birds	No. of dead birds / days post challenge										Total deaths	Protection % *	Reduction in viral shedding* (log_{10}/ml)
		1	2	3	4	5	6	7	8	9	10			
rFP-AI-H5 Scotland one dose	17				5	3	3	2	1			14/17	17.7	0.3
rFP-AI-H5 Ireland one dose	17	1			4	2	1	2	1			10/16	37.5	0.7
Inactivated H5N2 one dose	17	1					1		1			2/16	87.5	2.4
Control	10		4	6								10/10	0	0

* According the Standard Egyptian regulation for Quality Control, valid protection % is ≥ 80 % and valid reduction in viral shedding must be > 2 log_{10}.

Results of immune response from chickens vaccinated with two homologous doses of different types of AI vaccines

It was observed that in case of vaccination by two homologous doses from inactivated H5N2 vaccine, gave very high protection level reach 100% in comparison to the control group which show 100% mortality during the first 3 DPC. But chickens vaccinated with two homologous doses from rFP-AI-H5-Scotland and rFP-AI-H5-Ireland vaccines give protection level reach 23.5 and 47.1% as described in table (2).

Also, it was showed that there was reduction in local HPAI challenge virus replication either $10^{0.5}$ or $10^{1.3}$ EID_{50} from oropharyngeal swabs in case of chicken groups vaccinated with two homomlogous doses from rFP-AI-H5-Scotland and rFP-AI-H5-Ireland vaccines. But, two homologous doses from inactivated H5N2 vaccine gave $10^{3.9}$ EID_{50} reductions in shedding of the challenge virus from respiratory tract.

Table 2. Results of the efficacy of two homologous doses from different AI vaccines against challenge with local HPAI virus at 4 Week post the last vaccination.

Vaccine type	No. of birds	No. of dead birds / days post challenge										Total deaths	Protection %	Reduction in viral shedding (log_{10}/ml)
		1	2	3	4	5	6	7	8	9	10			
rFP-AI-H5 Scotland two dose	17				4	4	3	2				13/17	23.5	0.5
rFP-AI-H5 Ireland two dose	17			1	3	3	1	1				9/17	47.1	1.3
Inactivated H5N2 two doses	17											0/17	100	3.9
Control	10		2	8								10/10	0	0

Results of immune response from chickens vaccinated with two heterologous doses of different types of AI vaccines

It was observed that, 88.2% from challenged chickens vaccinated with two heterologous doses from rFP-AI-H5-Scotland and inactivated H5N2 don't show any signs of AI disease during 10 post challenge. At the mean time, vaccinated chickens with rFP-AI-H5-Ireland vaccine then boosted with inactivated H5N2 vaccine gave protection level reach 94.1%. However, the control group that received the same challenge dose, 100% chickens showed death with severe symptoms during 3 days PC as shown in table (3).

On the other hand, it was found the group of chickens vaccinated with rFP-AI-H5-Scotland vaccine and boosted by the inactivated H5N2 vaccine reduced the viral shedding in oro-pharyngeal swabs by 102.9 EID50. While, there were reduction in challenge virus shedding by 103.2 EID50 from oro-pharyngeal swabs in case of chickens group vaccinated with rFP-AI-H5-Ireland vaccine as primary vaccine and boosted with inactivated H5N2 vaccine.

Table 3. Results of the efficacy of two heterologous doses from different AI vaccines against challenge with local HPAI virus at 4 Week post vaccination

Vaccine type	No. of birds	No. of dead birds / days post challenge										Total deaths	Protection %	Reduction in viral shedding (log_{10}/ml)
		1	2	3	4	5	6	7	8	9	10			
rFP-AI-H5 Scotland + inactivated H5N2	17						2					2/17	88.2	2.9
rFP-AI-H5 Ireland + inactivated H5N2	17					1						1/17	94.1	3.2
Control	10		2	8								10/10	0	0

DISCUSSION

One of the major problems facing the poultry farms during last few years is the avian influenza disease which becomes common among poultry private and governmental farms *(7)*. Avian influenza viruses are important veterinary and human health pathogens around the world. It is unusual in that it can cause a range of disease symptoms in poultry from a subclinical infection to being highly virulent with 100% mortality *(10)*.

Vaccination to prevent or reduce losses due to AI disease is common. A variety of vaccines are therefore used in an attempt to control the disease, but it become evident that the most widely used methods of vaccination, did not always give adequate protection against the virulent local HPAI H5N1 virus. So, AI vaccines and their field application can be an effective tool within a control program which includes biosecurity, education, surveillance and diagnostics and the best method used to eliminate the AI virus infected poultry through the human euthanasia and environmentally sound disposal of carcasses or controlled marketing *(11)*.

The majority of AI vaccines used in the field are inactivated whole AI virus vaccines licensed for parented (subcutaneous and intramuscular) administration. Other types of live virus vaccines have been developed for AI using alternative In vivo expressed HA protein (live vector vaccines) as adenovirus *(12, 13)*, fowl pox virus *(14,15)*, baculovirus *(16-18)*, or Newcastle disease virus *(6,19,20)* and can provide some of the immunological advantages of a live virus vaccine but without reassortment risk of using a live AI virus *(3)*.

Challenge under strictly controlled conditions with virulent HPAI virus may also be used to predict flock response to AI exposure. This method can add considerable significance to test the immune efficacy of a vaccine. These findings substantiate the previous studies *(11,21)*. In this study, A / Chicken / Egypt / 1709-6 / 2008 (H5N1) was chosen as a challenge virus. The protection was evaluated by death and virus shedding from tracheal swabs. All the chickens immunized with two types of rFP-AI-H5-Scotland and Ireland as one or two doses were not protected against the challenge virus. The protection % of the chickens vaccinated with one and two doses of rFP-AI-H5-Scotland and rFP-AI-H5- Ireland vaccines were 17.7, 23.5, 37.5 and 47.1 %, respectively (Tab. 1 & 2). For the whole- virus inactivated vaccine, the protection % was 87.5 and 100 % when used as one or two doses. While the chicken of the control group showed 100% mortalities and suffered from severe clinical signs. Prevention of respiratory and general clinical signs (morbidity) and death (mortality) has been the most frequent used criteria to assess protection *(22,23)*.

But, from the data shown in Table 3 , it is observed that all groups of chicken vaccinated with two heterologous doses of different type of AI vaccines gave also a good protection against local HPAI virus. The chicken groups vaccinated with rFP-AI-H5-Scotland or Ireland and boosted with inactivated H5N2 vaccines evoked a degree of protection rate reach 88.2 and 94.1%, respectively.

From Tables 1 and 2, the present study, showed that there was greater reduction in local HPAI challenge virus replication either $10^{2.4}$ and $10^{3.9}$ EID_{50} from oropharyngeal swabs in case of chicken groups vaccinated with one dose and two doses of inactivated H5N2 vaccine, respectively, versus to the recombinant vaccines which produced very poor reduction in the titer of challenge virus. The superior protection may have resulted from proprietary adjuvant system, route and site of immunization and challenge virus dose *(24)*.

Meanwhile, as shown from Table 3, the reduction in the challenge viral shedding through oropharyngeal swabs treatment were 2.9 and 3.2 corresponding for groups of chicken vaccinated with two heterologous doses of rFP-AI-H5-Scotland or Ireland and boosted with inactivated H5N2 vaccines, respectively. In addition to prevention of disease and death, the prevention of infection or the qualitative and quantitative reduction in virus replication in respiratory and digestive tracts are essential protection criteria that indirectly assess the role of the vaccine to limit field virus spread and critical for control *(15)* .

Demonstration of a reduction in replication and shedding titers of virus from respiratory and intestinal tracts should be at a minimum of 10^2 EID_{50} (100-fold) less virus in vaccinated compared to non-vaccinated birds *(25,26)*. In addition, immunized birds have a quantifiable resistance to infection as measured by requiring 10^2 to 10^5 EID_{50} greater challenge dose to produce infection in vaccinated compared to non vaccinated bird *(27,28)*.

Finally, The obtained results illustrated that the using of inactivated H5N2 as 2 doses induced higher protection percentage and reduction in viral shedding, more than any of the other vaccines. One of the concerns of vaccination against AI is that single dose of current vaccines do not produce sufficient immunity to completely prevent infection and subsequent virus transmission, although recent experiments demonstrated that vaccination with inactivated vaccines may be able to reduce the spread of AIV within flock *(17,19)*. Also our it has been illustrated that inactivated whole influenza virus vaccine produce uniform protection of chickens from clinical signs and death following challenge by HPAI viruses *(15,25)*.

Also, the usage of the rFP-AI-H5 as prime-boost schedule vaccine with inactivated one in order to protect chicken flocks from avian Influenza infections is to somewhat more effective *(25,29)*.

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الملخص العربى

دراسات مقارنة بين رد الفعل المناعى للدجاج المحصن لكل من لقاح الأنفلونزا المحمل على فيروس جدرى الطيور ولقاح الأنفلونزا المثبط

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المعمل المركزى للرقابة على المستحضرات الحيوية البيطرية – العباسية - القاهرة

تم معايرة نوعين من اللقاح الحى لمرض الانفلونزا المحمل على فيروس جدرى الطيور احدهما محضر من العترة الايرلندية والاخر محضر من العترة الاسكوتلاندية ومقارنتهما باللقاح المثبط للانفلونزا وذلك باجراء اختبار التحدى و قدرتهم على حماية الطيور ضد الإصابة بفيروس الانفلونزا المحلى.

تم تحصين مجاميع من الدجاج الخالى من المسببات المرضية طبقاً لنظام معين من التحصين تم وضعه لدراسة مدى إستجابة الجهاز المناعى لهذه الطيور لتلك اللقاحات و كانت النتيجة كالاتى حيث اعطت مجموعة الدجاج المحصنة بجرعتين منفصلتين من اللقاح المثبط اعلى نسبة صد (١٠٠%) بينما استخدام جرعة واحدة منه اعطت نسبة صد (٨٧,٥%) فى حين ان اعطاء جرعة واحدة من اللقاح الحى المحمل على فيروس جدرى الطيور (الايرلندى او الاسكوتلاندى) يليه جرعة واحدة من اللقاح المثبط للانفلونزا قد اعطت نسبة صد (٨٨,٢%-٩٤,١%) على التوالى. بينما استخدام جرعة واحدة او جرعتين متتاليتين من اللقاح الحى للانفلونزا المحمل على فيروس جدرى الطيور قد اعطى نسبة صد ضعيفة (١٧,٧%, ٣٧,٥%, ٢٣,٥%, ٢٧,١%) على التوالى.

وفى النهاية وجد ان استخدام اللقاح المثبط كجرعة او جرعتين وكذلك استخدام اللقاح الحى المحمل على فيروس جدرى الطيور كجرعة اولية تليها جرعة من اللقاح المثبط قد استطاعوا جميعا التقليل من افرازات الفيروس الضارى بنسبة كبيرة بالمقارنة بباقى المجاميع.ايضا وجد ان اللقاح المحضر من العترة الايرلندية قد أعطى إلى حد ما نتيجة افضل من اللقاح المحضر من العترة الاسكوتلاندية.