PREPARATION OF INACTIVATED OIL ADJUVANT VACCINE FROM AI FIELD H5N1 VIRUS ISOLATE WITH IMMUNOSTEMULANT AS COMPARED WITH AVAILABLE COMMERCIAL VACCINES.

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SUMMARY

From a natural infected field cases with typical signs and lesion of HPAI virus H5N1 was isolated in SPF chicken embryos and identified by by HI test and confirmed to be HPAI H5N1 by NUMERO-3. A trial to prepare formalin inactivated AI vaccine from the isolated field strain in SPF chicken eggs. The inactivated fluid was added to oil adjuvant in rate of 1:1 the prepared vaccine proved to be sterile and safe as it pass the recommendation for safety and sterility by OIE-Manual (2004). The immunogenicity of that vaccine was tested by vaccination of 3 week old SPF chickens at the 3rd and 6th week of age and testing their sera for HI antibody titres against H5N1 antigen for 6 weeks.

Immunogenicity of the prepared vaccine was compared with imported H5N1 and

H5N2 vaccines in 7 weeks layer type chickens from non vaccinated flock. The imported vaccines resulted in HI titers higher than the prepared one (That needs improvement). The results can be attributed to the type of inactivator, antigen mass and oil adjuvant.

Addition of saponin to both prepared and imported vaccines resulted in higher HI antibodies than vaccine and levamisol and vaccine only. AI Imported vaccine with saponin showed positive results at the 1st wpv with titre of 0.50 ± 1.55, while other groups were still negative.

key words: Avian influenza, Immunity, Saponin, Levamisol, Immunostemulant, vaccine, HI-test.
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INTRODUCTION

Twenty-six AI epizootics of highly pathogenic AI have occurred in the world since, 1959 *(Alexander, 1986* and *Saif, et al., 2003).* The largest outbreaks has been caused by HPAI pathotype H5N1 virus, which caused a severe losses and problems in poultry industry and some wild birds in over 60 countries in Asia, Europe and Africa with human affections with deaths in 50% of affected cases (*Keawcharoen, et al., 2004, Thanawongnuwech, et al., 2005,* and *Webster, et al., 2006).*

Aquatic birds, particularly ducks, shore birds, and gulls, are considered the natural reservoirs for AI viruses *(Webster, et al., 1991* and *FAO/OIE/WHO-Reports, 2006/ 2007).*

These birds generally do not developed disease when infected *(Horimoto and Kawaoka, 2001* and *Webster, et al., 2006);* however, an outbreak of H5N1 was identified in migratory geese and other wild birds in Qinghai, China, May 2005. Asymptomatically infected domestic ducks are shedding more H5N1 virus for longer periods *(WHO-Reports, 2004).*

The AI 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 (*Chen, et al., 2006* and *Webster, et al., 2006).*

Prevention of AI passed on strategies by *FAO-September, (2004)* as bio-security 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. In endemic areas, vaccination of poultry flocks by inactivated 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 (*Zanella,et al., 1981, Luschow, et al., 2001; FAO-September, 2004* and *Van der Goot, et al., 2007*).

This study was planed for preparation of inactivated oil adjuvant vaccine from isolated AI field H5N1 virus. Usage of saponin or levamesol as immunostemulant for improve immune response of prepared vaccine as compared with available commercial H5N1 vaccine.

MATERIAL AND METHODS

Avian influenza virus isolates:

HPAI-H5N1 isolated clinically diseased and dead chicken from the AI current outbreak in Egypt during May/2006. The virus was identified by Namero-3 as HPAI-H5N1.

Suspected chickens were samplled for virus isolation. About 2 grams out of liver, trachea and intestineal content were aseptically collected and kept at -20 C for isolation of AI.

Embryonated chicken eggs (ECEs)**:**

Specific pathogen frees (SPF) obtained from Kom Oshim, El-Fayoum, Egypt. ECEs were used for virus isolation and propagation of isolated virus vaccine and HI antigen.

Chicks and chickens:

I. SPF chickens: Five, 9-week old SPF chickens for preparation of immune serum for the H5 antigen.

2. Fifteen 3 weeks old SPF chickens, for safety and immunogenicity of prepared vaccine.

3. **Commercial chicks:** Two hundred and seventy fife layer chicks for comparing immuno-genicity of prepared and imported H5N1 and H5N2 vaccines as well as testing of immuno-stimulant effect.

Serum and known antisera:

Positive antisera: Known positive Newcastle disease virus (NDV) immune serum was obtained from the department of Newcastle Disease, VSVRI, Abassia, Cairo.

Positive H5-AIV serum:
Positive H5-AIV serum for locally isolate was prepared in 9-weeks old SPF chickens according to *Herbert, (1976), and Abd El-Wanis, et al., (2002)*.

Avian influenza vaccines:
Inactivated H5N1 subtype, Re-1 Strain Vaccine was obtained from VACSERA, Egypt. The vacccine is type A, A/ Harbin /Re-1/2003 (H5N1). in a mineral oil adjuvant for intra-muscularly in a dose of 0.5 ml/bird, The vaccine was produced by Harbin Weike Biotechnology Development Co,, China. License No.: (2005) 080012098. AI-H5N2 inactivated vaccine produced by Intervet Corporation.

Wahed Red blood cells (RBCs):
Washed chicken RBCs was prepared in PBS in concentration of 10 % for rapid HA and 0.75 % in micro-HI-test according to ***Anon, (1971).***

Immuno-stimulants:
a. Saponin: Saponins as an Immuno-stimulant (IS) ingredient (component) in the inactivated vaccines in animals *(Ekbal, 2005)* and in Birds by *Isconova (2005)*. Purified, Sigma Prod. No. S4521, CAS Number: 8047-152.
b. Levamizole: Levamizole From VET WIC, EL Nasr Pharmaceutical Chemicals Co., Vet. Div., Egypt. Levamisole HCL, Reg. No. : 752, Batch No. 1/908/05.

Material used for vaccine preparation:
Oils (Adjuvants): White Paraffin oil, Tween 80 and Span oils. White Paraffin oil: (White x 300) white oil quality FDAIAL usp No. 05200 Mobil.
Aquous phase emulsifier (Tween 80): poly oxyethelene sorbitan, supplied by Sigma (HLB=15). Oil phase emulsifier Arlacil (Span 80): Sorbitan Monooleate supplied by Micbile, Aleyandrid (HLB=4.3). The following chemicals were used Glycine (NH2-CH2-COOH) produced by El-Nasr pharmaceutical Co., B. No. 2006/1, and was used as 4.8 gm/Liter of vaccine. Sodium Thiomersal: 1/10 solution, and was used as 1 ml / 1 Liter vaccine AD3E oily vitamin produced by Arabcomed Company, Egypt.

Inactivation of AI - virus:
Commercial formalin solution 40% was obtained from El-Nasser chemical Co, Egypt was used in 0.1 % final concentration according to *Beard, (1989) and Abd El-Wanis, et al., (2004)* and 0.5 % to inactivate AI virus for vaccine and antigen *(Marandi and Fard, 2001).*

Egg inoculation:
The procedures of sample preparation and egg inoculation were done according to *Allan (1981)* and *Pearson and Senne (1981)*. Slide HA was applied on allantoic fluid of inoculated chicken embryos to detect. Estimation of 50% end point was carried out according to *Reed and Muench (1938).*

hemagglutination (HA) tests:
A- Rapid slide HA tests: Standard method described in *Anon (1971)* was used for quick detection of haemagglutinin in embryonic fluid.

B- Plate HA tests: The HA test was carried out in V-bottomed microtitre plate following the recommendation of *OIE-*

Manual, (2004). The test was used for identification of isolate and detection of 4HA Units of used antigens.

Hemagglutination inhibition (HI) test:
The HI test was adopted according to ***OIE-Manual, (2004)*** in V-bottomed microtitre plate and 4 HAU of virus/antigen in 0.025 ml. Phosphate buffer saline (PBS) was prepared according to ***Cruickshank et al., (1975).***

Preparation of inactivated Vaccine AI:
All the methodology and steps of inactivated vaccine preparation were done according to *Stone, (1987; 1988; 1991 and 1993).* Virus titre was $10^{7.5}$ EID_{50}/ml before inactivation.

Sterility, infectivity and immunogenicity of prepared H5N1 vaccine:
The prepared vaccine was tested for sterility, infectivity and immunogenicity according to the OIE-Manual (2004):
1- Infectivity and safety: The inactivated aqueous phase was passaged serially 3 times in allantoic sac of 9 days old SPF-ECEs, where the allantoic fluid gave negative HA results in the all 3 passages.
2- Sterility: The prepared vaccine was cultured on Sabouraud Dextrose Agar, Nutrient Agar and Mycoplasma Media for sterility.

3- Immunogenicity and duration of immunity in SPF chicks:
Fifteen SPF, 21-days old SPF-chicks were used as 10 birds were inoculated at the 3rd and 6th week of age, while the other 5 birds were kept as non-inoculated control. The birds were kept under daily observation for 7 weeks with the collection of individual weekly blood samples for serum. The collected sera were tested against H5 antigen using the HI test (table 1).

Blood sampling for sera:
Blood samples for serum were collected from jugular vein or by slaughtering of the 1- days old birds (*Jain, 1986*). The sera were collected in dry sterile tubes and stored at – 20 C^o till use**.**

Comparative study on immunity of prepared and commercial vaccine:
One hundred, 7 weeks layer type chickens from non-vaccinated flock and negative to AI-HI antibodies were used. The chickens were randomly divided into 4 groups (1- 4); 25 chickens each. The chicken of groups 1, 2 and 3 were s.c injected with imported H5N1 vaccine, prepared vaccine, and imported H5N2 vaccine; respectively while birds of group 4 were kept as non-vaccinated control. All vaccines were given in dose 0.5 ml S/C in the back of the neck. Blood samples for serum were weekly collected from the 1st to the 8th week post vaccination for detection of HI antibodies against AI-H5 antigens. The obtained results were presented in table (2).

Trials to Improve quality of AI vaccine using immuno-stimulants
One hundred seventy five, 1 day old layer type chicks were obtained from non vaccinated commercial flocks. The chicks were floor reared and fed on commercial ration. At the 3rd week of life 20 blood samples were taken randomly for serum and proved to be negative for the HI test against the AI-H5 antigen.

In this experiment saponin or levamizole was added separately to the prepared and imported vaccines as immuno-stimulant. Then chickens were divided into 7 groups (1-7); 25 chickens each, and treated as the follows: Group (1) was given prepared vaccine. Group (2) was given prepared vaccine + Saponin. Group (3) was given prepared vaccine + Levamizole. Group (4) was given imported vaccine. Group (5) was given imported vaccine + Saponin. Group (6) was given imported vaccine + Levamezole. Group (7) was kept as non-vaccinated control.

All chicken groups were kept under daily observation for 4 weeks with collection of weekly blood samples for sera to determine AI antibodies using HI test against the H5 antigen. The obtained results are presented in table (3).

RESULTS

No embryo mortality was observed from passage of inoculated inoculums as well as no HA activity was detected at the 6th day post-inoculation indicating safety of the inactivated virus. No fungal or bacterial growth could be detected indicating purity of the prepared vaccine.

The inoculated SPF chicks showed no clinical signs or mortalities allover the observation period, and the HI titers are 0, 2.00, 4.00, 5.00, 6.71 and 6.85 at 1, 2, 3, 4, 5 and 6th week post-inoculation, While there were no positive serum results in the non-inoculated control birds.

Table (1): HI titers in SPF chickens received prepared vaccine.

Weeks Post vaccination	Vaccinal dose	HI titer distribution							Mean ± SD
		0	2	3	4	5	6	7	
1	1st	8							0.00 ± 0.00
2		1	4	2					2.00 ± 1.00
3				1	4	1			4.00 ± 1.75
4	2nd				1	4	1		5.00 ± 0.44
5							2	5	6.71 ± 0.48
6							1	5	6.85 ± 0.40

No clinical signs or mortalities were seen allover the experimental in all groups. All vaccinated groups showed HI test in the 3rd week, Except those of group 3 received the Imported H5N2 where the mean titres was 1.50±1.64 and 3.10±1.46 at the 1st and 2nd wpv; respectively. Birds of group 3 showed the highest titers at all times with the maximum titres of 8.00 ± 0.89 at the 4th and 5th wpv, followed by those of group 1 given

Imported H5N1 vaccine (6.00 ± 0.95 and 5.00 ±1.15 at the 5th and 6th wpv) and finally in group 2 that received the locally vaccine that showed the lowest but gradually increased titres to reach 4.00 ± 0.63 at the 8th wpv.

Table (2): HI titers in vaccinated chickens different AI vaccines.

Group	vaccin	Dose	W pv	Distribution of HI titre (TRN)								Mean - ± SD	
				0-2	3	4	5	6	7	8	9		
1	Imported H5N1	1/2 ml	1	7								0.00	0.00
			2	7								0.00	0.00
			3	1	5	1						2.71	1.25
			4	1		2	4					3.50	1.83
			5			3	2	1	1			5.00	1.15
			6				2	1	4			6.00	0.95
			7					2	5			6.00	0.45
			8	1	1		2	3				4.44	1.78
2	Local H5N1	1 ml	1	6								0.00	0.00
			2	6								0.00	0.00
			3	2	4							2.00	1.34
			4	1	5							2.50	1.22
			5	1		4	1					3.16	1.76
			6	1	5			1				3.00	1.73
			7		3	1	1					3.00	0.89
			8		1	4	1					4.00	0.63
3	Imported H5N2	1/2 ml	1	3	3							1.50	1.64
			2	1	2	4						3.10	1.46
			3	1		2	2	1				6.00	2.09
			4						2	2	2	8.00	0.89
			5					1		4	1	8.00	0.98
			6						1	6		7.00	0.37
			7	1						6		6.85	3.02
			8				1	2	5			5.50	0.75
4	Control		1--8	6								0.00	0.00

Addition of immuno-stimulant to both prepared and imported vaccines resulted in higher antibodies to AI than vaccine only as measured with HI-test (table 3 and Fig 3 and 4).

Imported vaccine with saponin (group 5) showed positive results at the 1st wpv with titre of **0.50 ± 1.55**, while other groups were still negative. Saponin with both vaccines (group 2 and 5) resulted in higher titres than levamizole. Saponin resulted in more hemogenous titres (lower SD values) than levamizole and vaccine only. Saponin groups showed the titres > 4 (near the protective) at the 4th wpv, while those received vaccine only or with levamizole still lower.

Table (3): The effect of immune stimulants on the HI titer of vaccinated chickens with prepared or imported AI-H5N1 vaccines (n = 6).

Group No	vaccine	immuno-stimulant	WPV	Distribution of HI titre (TRN)				M. ± SD
				0-2	3	4	5	
1	prepared H5N1	-	1	6				0.00 0.00
			2	4	2			1.00 1.55
			3	2	4			2.00 1.55
			4	1	5			2.50 1.22
2		Saponin	1	6				0.00 0.00
			2	3	3			1.50 1.48
			3		3	3		3.50 0.55
			4			5	1	4.17 0.44
3		Levamizol	1	6				0.00 0.00
			2	4	2			1.00 1.55
			3	1	4	1		2.80 1.37
			4		6			3.00 0.00
4	Imported H5N1	-	1	6				0.00 0.00
			2	5	1			0.50 1.55
			3	1	4	1		2.66 1.66
			4			2	4	4.50 0.52
5		Saponin	1	5	1			0.50 1.55
			2		2	4		3.83 0.54
			3		1	5		3.00 0.41
			4		1	4	1	4.00 0.63
6		Levamizol	1	6				0.00 0.00
			2	4	2			1.00 1.48
			3		3	3		3.83 1.55
			4		3	3		3.83 1.55
7	Control		1- 4	6				0.00 0.00

Discussion

The isolation and identification of AI virus from affected chicken flock reared in Qualubia governorate, at March 2006 showing signs of high mortality, sudden stopped egg production, marked cyanosis in the comb and haemorrhages in shanks. The post-mortem examination revealed that marked congestion in tracheas and subcutaneous blood vessels as well as internal organs. These signs and lesions are similar to those caused by HPAI virus *(CEC, 1992; Alexander, 2003; Saif, et al., 2003; Swayne and Halvorson, 2003, OIE-Manual, 2004* and *Fouchier, et al., 2005).*

The collected samples were passed in SPF chicken embryos and showed specific embryo mortalities with lesions of AI as stated by *(Saif, et al., 2003).* The virus was identified to HA activity according to *OIE-Manual, (2004)* and serologically tested against ND serum by HI test. HI test shown

to be effective in as an effective serological method for identification of AIV isolates *(Jorgensen, et al., 1998; Manvell, et al., 2000; Woolcock, et al., 2000; OIE-Manual, 2004* and *Fouchier, et al., 2005).* Moreover; As HI test had been recommended and proved to be effective as a serological test for monitoring of AIV antibodies and evaluation of immune response in the vaccinated chickens by *Naeem, et al., (2003)* used the HI tests in monitoring sero-prevalence of avian influenza virus and its relationship with increased mortality and decreased egg production. *Lu and Castro, (2004)* used the HI tests in evaluation of the immune response of a low-pathogenicity AI-H7N2 virus in specific-pathogen-free chickens. *OIE-Manual, (2004)* reporting the HI titres may be regarded as being positive if there is inhibition at a serum dilution 2^4 or $\log_2$ 4 or more against 4 HAU of antigen. *Tang, et al., (2005)* used the HI test as an effective serological method. The collected embryonic fluid was sent to NAMRU-3 proved that the isolated AI strain was HP-H5N1.

As a trial to prepare inactivated AI vaccine from the isolated field strain through passage in SPF chicken eggs and inactivated with formalin as stated by *Daubney et al., (1949) and Eissa, (1960).* On the other hand formalin treated allantoic fluid was resulted to have reduction in the HI activity of virus as it dropped from $\log_2$ 5 to 3 – 4. This result clear the harmful effect of formalin in the HA antigen of AI virus as stated by *Goldstein and Tauraso, (1970)* and *Bahnemann, (1997)* reported that formalin as an in-activator for viruses like AIV, has been used from many years. *El-Bishlawy, (1945)* found similar results in using formalin with AI vaccine.

The effect of formalin is exerted on the surface proteins and its effect on the nucleic acid occurs after complete damaging of the surface proteins The inactivated fluid was added to the oil adjuvant in rate of 1:1 This prepared vaccine proved to be sterile and safe as it pass the recommendation for safety and sterility by *OIE–Manual, (2004).* The immuno-genicity of the prepared vaccine was tested by vaccination of 3 week old SPF chickens at the 3rd and 6th week of age and testing their sera for HI antibody titres against H5N1 antigen for 6 weeks. The obtained results indicating the detection of HI antibodies titers 0, 2.00, 4.00, 5.00, 6.71 and 6.85 at 1st , 2nd , 3rd , 4th , 5th and 6th wpv in sera of vaccinated SPF chickens (table 1).

Finally to use the local HPAI strain in production of vaccine it needs to be reassorted or concentrated to increase its antigen-mass contents, or using a hetrologous H5 LPAI strain like LPAH-H5N2 strain to be used in preparation of vaccine of high antigen-mass content due to the disadvantages of using the HPAI viruses in vaccine production are the very low antigen-mass contents besides the great public hazards, the can also improve its immuno-genesity using different immuno-stimulants.

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تحضير لقاح مخمد زيتى من عتره حقلية من فيروس انفلونزا الطيور ه1 ن1
مع منشط مناعى ومقارنته اللقاحات التجارية.

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الملخص

تم عزل فيروس إنفلونزا الطيور عالى الضراوة من حالات إصابة الطيور الحقلية مع معدلات نفوق وأعراض وتشريح بمحافظة القليوبية فى أجنة بيض الدجاج الخالى من المسببات المرضية (SPF Eggs) وتم التعرف على الفيروس بعد استبعاد فيروس النيوكاسل بإختبار مانع تلازن الدم (HI test) وأرسلت المعزولة الى وحدة بحوث النواميرو- 3 حيث عرفت وحللت وراثيا وسجلت كعترة شديدة الضراوة للفيروس HP-H5N1 .

تم تحضير لقاح فورمالينى زيتى مثبط من العترة المعزولة محليا, وأثبتت الدراسة ان اللقاح المحضر امن وقادر على إحداث المناعة فى الدجاج الخالى من المسببات المرضية عمر 3 اسابيع وتم تتبع الأجسام المناعية لمدة 6 أسابيع من الحقن , تمت مقارنة القدرة المناعية للقاح المحضر باللقاحت التجارية المستوردة من النوع المصلى H5N1 and H5N2 فى دجاج بياض تجارى غير محصن عمر 7 أسابيع حيث أوضحت المقارنة أن اللقاح التجارى أعطى معدلات اجسام مناعية (HI titers) أعلى من المحضر محليا وأرجع ذلك لنوع المادة المثبطة , كمية المستضاد (الأنتيجن) , نوع الزيت والتى تستلزم إمكانيات عالية للتعرف عليها.

أستخدمت مادة الصبونين كمحفز مناعى لكل من اللقاح المحضر من العترة المحلية واللقاح المستورد وأدى الى إحداث مستويات مناعية إبتداء من الأسبوع الأول بعد الحقن وأعلى فى كل العينات الأسبوعية اذا ماقورنت بنتائج المجموعات المحصنة باللقاح مع الليفاميزول او اللقاح فقط .