# Trials for preparation of inactivated avian influenza vaccine from local isolate H5N1

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## ABSTRACT

In the present study avian influenza (AI) inactivated vaccine was prepared with Montanide ISA70VG which has been added as adjuvant using reassortant AI strain H5N1. The prepared vaccines with Montanide ISA70VG varied in the HA units per dose and also differed in the ratios between two reassortant strains (Chicken and duck strains) of AI. The results of testing humoral immunity revealed that the prepared vaccines with Montanide ISA70VG containing 350 HA unit/dose were high in antibody titer than vaccines containing 256 and 100 HA unit/dose. The prepared vaccines with Montanide ISA 70VG containing 256 HA unit/dose gave high antibody titer than vaccines containing 100 HA unit/dose. The ratio of 30% Chicken strain of AI to 70 % Duck strain of AI gave the best result of antibody titer followed by the ratio 40% to 60% followed by 50% to 50% respectively. The highest titer observed in the prepared vaccine was in the ratio of 30% Chicken strain of AI to 70% Duck strain of AI containing 350 HA unit/dose after 3 weeks post vaccination and remained high till 30 weeks post vaccination with protective antibody titer and protection against challenge followed by the ratio of 30% Chicken strain to 70 %Duck strains of AI containing 256 HA unit/dose which gave high antibody titer after 4 weeks post vaccination and remained high till 26 weeks post vaccination followed by the ratio of 30% Chicken strain to70 %Duck strain of AI containing 100 HA unit/dose which gave high antibody titer 5 weeks post vaccination and remained high till 20 weeks post vaccination. The results of testing cell mediated immunity revealed that the prepared vaccines containing 350 HAU/dose induced significant high lymphocyte cells followed by 256 HAU/dose followed by 100 HAU/dose. The challenge test revealed that the prepared vaccine in the ratio of 30% Chicken strain of AI plus 70% Duck strain of AI containing 350, 256 and 100 HA unit/dose showed protection reached to 85%, 80% and 30% respectively. In conclusion, the study highlight the add value of using different HA units/dose of AI and different ratios of the two strains (chicken and duck strains).

## INTRODUCTION

Avian Influenza (AI) is an infectious disease of birds caused by specified viruses that are members of the family Orthomyxoviridae and placed in the genus influenzavirus A (Voyles, 2002). Influenza viruses type A are enveloped negative-sense, segmented, single-stranded RNA viruses. They have antigenically related nucleocapsid and matrix proteins, but are classified into subtypes on the basis of their haemagglutinin (H) and neuraminidase (N) antigens (Suarez et al., 2004). At present, 16 H subtypes (H1–H16) and 9 N subtypes (N1–N9) are recognized. To date, the highly virulent influenza A viruses that produce acute clinical disease in chickens, turkeys and other birds of

economic importance have been associated only with the H5 and H7 subtypes. Most viruses of the H5 and H7 subtype isolated from birds have been of low virulence for poultry (Fouchier et al., 2005). As there is the risk of a H5 or H7 virus of low virulence becoming virulent by mutation, all H5 and H7 viruses have been identified as notifiable AI viruses (OIE. 2008).

In Egypt, since the first isolation of avian influenza virus H7N1 from turkeys by Khafagy et al. (1992), non of the H7 or H5 subtypes have been isolated. Egypt confirmed its first H5N1 outbreak in poultry on 17 February 2006 in three governorates: Cairo, Giza, and Al-Menya, soon the disease was deeply entrenched in poultry population all over the country. The larger number of outbreaks in poultry occurred between March and April 2006. Although since the end of June 2006 there were no poultry farms involved, several infection foci were being reported in poultry from backyards, roof tops and live bird markets. This suggests that despite the strenuous control measures the virus may be becoming endemic in the country (WHO, 2006). This virus spread was attributed to Free ranging backyard chickens and ducks, illegal transportation of birds as well as infected migratory waterfowl (Tiensin et al., 2005; Webster et al., 2006).

Vaccination with inactivated AI virus vaccines were found to be an effective mean to lower losses from mortality, reduce the viral load in the environment and risk of human infection as well as eradication of positive cases in endemic area (Van der Goot et al., 2005). Conventional inactivated whole AI virus vaccine is usually prepared as homologous (contain the same AI virus strain as the one causing the problem in the field) or heterologous (differ in that the virus strain used in the vaccine is of the same H type as the field virus but has a heterologous neuraminidase). This vaccine is usually prepared from low pathogenic virus (Capua and Marangon, 2003).

The progress in vaccination is directed towards the selection of the proper adjuvant that can elaborate high and long lasting immunity. So adjuvants considered as one of the important factors in vaccine formulation due to it can prolong the immune response and stimulate specific components of the immune response either humoral or cell mediated immunity (SEPPIC, 2002).

The ideal adjuvant should increase a vaccine's immunogenicity without adversely affecting the safety of the immunogen. Some adjuvants have failed because they were associated with unacceptable toxicities, even though they led to significantly improved immune responses, as formation of sterile abscesses at the site of injection (Aguilar and Rodriguez 2007).

The present study was designed to spot the light on the immunogenicity of prepared inactivated reassorted avian influenza virus (H5N1) vaccine supplemented with Montanide ISA70 as adjuvant in chickens.

## MATERIAL AND METHODS

### 1. Avian Influenza (H5N1) reasortant strains:

A reassortant Avian Influenza virus A/Chicken/Egypt/Q1995D/2010 (H5N1) 1010EID50 /ml and 10 Log2 HA activities and A/Duck/Egypt/M2583D/2010 (H5N1) of a titer 1011EID50 /ml and 11 Log2 HA activities was provided to Veterinary Serum and Vaccine Research Institute, Newcastle disease unit, Abbasia, Cairo by the United State Department of

Agriculture (USDA). It was used for preparation of inactivated AIV vaccine with different adjuvants and HI test.

**2. Experimental chicks:**

Eight hundred and fifty (850), one day-old SPF chicks were purchased from SPF poultry project, kom oshim, EL-Fayoum Governorate. They were floor reared, fed on commercial poultry ration, and kept under strict hygienic measures throughout the experiment. The chicks were used for studying the safety and evaluating of the prepared vaccines.

**3. Embryonated Chicken Eggs (ECE):**

Fertile specific pathogen free embryonated chicken eggs (SPF – ECE) were purchased from the specific pathogen free egg project, kom oshim, EL-Fayoum Governorate. The eggs were incubated at 37°C and 80% humidity until inoculated at 9-11 days of age via allantoic sac route. They were used for preparation of the vaccinal patch, titration of the vaccines and testing the safety of prepared inactivated virus suspension.

**4. Serum samples:**

Serum samples were collected from all chicks (vaccinated and non-vaccinated) weekly till 8th week post vaccination then every 2week till the 30th week post vaccination and lastly at the 36th week post vaccination. The sera were inactivated at 56°C for 30 minutes, and then stored at -20°C until used in HI test.

**5. Cell Viability Assay Kit (md biosciences, USA) :**

The kit was used in the lymphocyte blastogenesis assay

6. Adjuvants:

Montanide ISA 70 VG, It was obtained from SEPPIC, Cosmetics, Pharmacy Division, Paris, France. NO 948400. It comprises a high grade injectable mineral oil and an extremely refined emulsifier obtained from mannitol and purified oleic acid of vegetable origin. Montanide ISA 70 is free from animal origin ingredients.

**7. Vaccine preparation:**

Reassortant Avian Influenza A\Chicken/Egypt/Q1995D/2010 (H5N1) and A/Duck/Egypt/M2583D/2010 (H5N1) was propagated on 9-11 days old specific pathogen free-embryonated chicken eggs (SPF – ECE) according to Garcia et al. (1998). Seventy two, SPF-ECE were inoculated with 0.2 ml/egg AIV (H5N1) via the allantoic sac, and then incubated at 37°C and 80% humidity for 5 days. Inoculated eggs were candled twice daily and dead embryos during the first 24 hours after inoculations were considered as non- specific deaths and discarded. Dead embryos or embryos close to death as well as live embryos (5 days post inoculation) were chilled overnight at 4°C until harvesting time. Eggs were opened under aseptic conditions and the allantoic fluid harvested and tested for HA activity using chicken RBCs suspension. Allantoic fluid of all eggs was collected aseptically and tested for sterility as well as titration of the virus content. Five SPF-ECES were used as control (non-inoculated).

**8. Titration of the virus in harvested allantoic fluid:**

Serial tenfold dilutions of the virus were prepared in PBS to which antibiotic solution was added. From each viral dilution 0.1 ml was inoculated into five SPF-ECES via the allantoic sac and the egg sealed and incubated at37c̊ , 80% humidity for five days. The eggs were incubated at 37c̊ , candled daily for 5 days and deaths within first 24 hours post inoculation were considered as nonspecific deaths. During and at the end of incubation period dead and survived embryos were chilled for 24hr at 4oc before harvesting. Haemagglutination test (HA) was used to detect the end point and EID50 was calculated according to (Reed

**Concentration and Inactivation of A.I Virus:**

Concentration of the virus using polyethylene glycol 6000 was purchased from Loba Chemie (India). Inactivation of the propagated virus suspension with formalin; the inactivation process was carried out according to (Beard, 1989). Twenty five ml of formalin solution 10% was added to each liter of the virus fluid; stirring was pursued during inactivation process. Samples from the virus formalin mixture were collected every 3 hours till 12 hour then collected every 1 hour till 24 hour in a screw capped tube for virus titration and HA activity. Finally, sodium bisulfite was added to a final concentration of 2% to neutralize formalin residues. To assure completion of virus inactivation, Samples from the inactivated virus harvest should be tested by at least two passages in 9-11 day old SPF embryonated eggs (0.1 ml /egg) via the allantoic cavity and tested by the rapid slide haemagglutination test as described by Anon (1971).

**9. Preparation of inactivated H5N1 AIV vaccine adjuvanted with Montanide ISA-70** Oil Adjuvant:

It was prepared as water in oil emulsion (W/O) using Montanide ISA70 V at a ratio of 3 / 7 (v/v) aqueous /oil ratio. Manufacturing process was carried out according to the standard protocol of SEPPIC and manufacturer instruction.

**10. Quality control of the prepared vaccines:**

Experimental batches of the prepared vaccines were tested for its sterility and freedom from any fungal or bacterial contaminants by culturing on specific media. For safety testing of the experimental vaccine batch, four groups (each composed of 10 chicks, 3 weeks old) were inoculated with 2 field doses (1ml) of the prepared vaccine at the nap of the neck. Another group of chicks were left as control (non inoculated). These chicks were observed for 2 weeks for any signs of local reaction or appearance of any clinical signs. After 5 days of inoculation, some birds were subjected to post mortem examinations to detect any pathological lesions.

## RESULTS

**1. Propagation and titration of AIV (H5N1) on SPF ECEs:**

The reassortant avian influenza virus A\Chicken\Egypt\Q1995D\2010 (H5N1) and A/Duck/Egypt/M2583D/2010 (H5N1) was propagated on 72 SPF-ECEs through allantoic cavity. Figures depicted in table 1 point out to the highest infectivity titer reached for AIV virus A\Cicken\Egypt\Q1995D\ 2010 (H5N1) was 10 log10 EID50/ml and 11 log10 EID50/ml for A\Duck\Egypt\M2583\2010(H5N1) virus .

**2. Inactivation of AIV (H5N1) with 0.1%formalin:**

The egg adapted AI virus A\Chicken\Egypt\Q1995D\2010 (H5N1) and A/Duck/Egypt/M2583D/2010 (H5N1) was inactivated by using 0.1% formalin solution according to Beard (1989). It was observed that the infectivity of the virus was completely diminished after 18hr from treatment table (3).

**3. Completion of inactivation:**

Inactivated AI virus A\Chicken\Egypt\Q1995D\2010 (H5N1) and A/Duck/Egypt/M2583D/2010(H5N1) prepared suspension were inoculated into 9 days old specific pathogen free embryonated chicken eggs (SPF-ECEs) through the allantoic sac and examined daily for 6 days. Results showed that there were no any pathological lesions, HA

activity and/or deaths of inoculated embryos.

**4. Quality control tests of the prepared inactivated AIV (H5N1) vaccines.**

**a. Sterility test:**

The prepared inactivated vaccines were cultured on different synthetic media for detection of bacterial and fungal growth. It was found that, the vaccines were sterile as they were free from any bacterial and fungal contaminants, as shown in table 4.

**b. Safety test:**

Different prepared inactivated vaccines were inoculated in 3 weeks old chicks through S/C route (1ml / chick) at the nap of the neck and examined daily for 2 weeks. It was observed that, there were no local or systemic reactions and also, no mortality in inoculated chicks, as shown in table 3.

**Table 1: Titration of AIV (H5N2) on SPF-ECEs**

| HA Titer ($log_2$HAU/1ml) | Infectivity Titer ($log_{10}$EID50/1ml) | Propagated virus |
|---|---|---|
| 10<br>11 | 10<br>11 | A\Cicken\Egypt\Q1995D\ 2010 (H5N1).<br>A/Duck/Egypt/M2583/2010 (H5N1) |

**Table 2: Sterility test of the prepared inactivated AIV (H5N1) vaccines.**

| Result | Examined micro-organism | Medium |
|---|---|---|
| No colonies | Aerobic bacteria | ***Nutrient agar*** |
| Clear (no turbidity) | Anaerobic bacteria | ***Thioglycolate broth*** |
| No colonies | Fungus | ***Sabarouds-agar*** |

**Table 3: Safety test of the prepared inactivated AIV (H5N2) vaccines.**

| Signs observed in 3 week old chicks for 2 weeks | | | Prepared vaccines |
|---|---|---|---|
| ***Chick mortalities*** | ***Systemic reaction*** | ***Local reaction*** | |
| Negative | Negative | Negative | **Groups of 100 HA unit/dose** |
| Negative | Negative | Negative | **Groups of 256 HA unit/dose** |
| Negative | Negative | Negative | **Groups of 350 HA unit/dose** |
| Negative | Negative | Negative | ***Control*** |

* AIV-$ISA_{70}$: Inactivated AIV (H5N1) with montanide $ISA_{70}$ oil adjuvant vaccine.

**5. Efficacy of inactivated AIV vaccine: Evaluation of cellular immune response to Inactivated H5N1 AIV adjuvanted with Montanide ISA70 oil adjuvant: Lymphocyte blastogenesis**

Cell–mediated immune response were evaluated for chicks vaccinated with Inactivated AIV (H5N1) with montanide ISA70 oil adjuvant vaccine by lymphocyte blastogenesis with kit using XTT reagent and without kit. It was noticed that, significant cell proliferation expressed by optical density was induced by AIV-ISA70 compared with that of control one in the 3 groups of the chicken and duck strains in ratio (30:70) as shown in tables 4,5,6,7 and 8.

**Table 4: Lymphocyte Blastogenesis of Chickens Vaccinated Inactivated AIV adjuvanted with ISA-70 Adjuvant:**

| Cell proliferation expressed by optical density | | | | days post vaccination |
|---|---|---|---|---|
| control | | AIV- ISA70 | | |
| Without XTT Reagent | With XTT Reagent | Without XTT Reagent | With XTT Reagent | |
| **0.189** | **0.375** | **0.308** | **1.224** | **5th day in group 100 HA units/dose** |
| **0.244** | **0.316** | **0.265** | **1.263** | **5th day in group 256 HA units/dose** |
| **0.142** | **0.344** | **0.401** | **1.300** | **5th day in group 350 HA units/dose** |

**Table 5: Lymphocyte Blastogenesis of Chickens Vaccinated Inactivated AIV adjuvanted with ISA-70 Adjuvant:**

| Cell proliferation expressed by optical density | | | | days post vaccination |
|---|---|---|---|---|
| control | | AIV- ISA70 | | |
| Without XTT Reagent | With XTT Reagent | Without XTT Reagent | With XTT Reagent | |
| **0.111** | **0.344** | **0.250** | **1.084** | **7th day of group 100 HA unit/dose** |
| **0.233** | **0.322** | **0.223** | **1.093** | **7thday of group 256 HA unit/dose** |
| **0.121** | **0.301** | **0.301** | **1.112** | **7th day of group 350 HA unit/dose** |

**Table 6: Lymphocyte Blastogenesis of Chickens Vaccinated Inactivated AIV adjuvanted with ISA-70 Adjuvant:**

| Cell proliferation expressed by optical density | | | | days post vaccination |
|---|---|---|---|---|
| control | | AIV- ISA70 | | |
| Without XTT Reagent | With XTT Reagent | Without XTT Reagent | With XTT Reagent | |
| **0.074** | **0.147** | **0.326** | **0.942** | **10th day of group 100HA unit/dose** |
| **0.080** | **0.097** | **0.407** | **1.157** | **10thday of group 256 HA unit/dose** |
| **0.037** | **0.125** | **0.393** | **1.159** | **10th day of group 350 HA unit/dose** |

**Table 7: Lymphocyte Blastogenesis of Chickens Vaccinated Inactivated AIV adjuvanted with ISA-70 Adjuvant:**

| Cell proliferation expressed by optical density | | | | days post vaccination |
|---|---|---|---|---|
| control | | AIV- ISA70 | | |
| Without XTT Reagent | With XTT Reagent | Without XTT Reagent | With XTT Reagent | |
| **0.073** | **0.082** | **0.149** | **0.534** | **15th day of group 100HA unit/dose** |
| **0.052** | **0.099** | **0.179** | **0.601** | **15thday of group 256 HA unit/dose** |
| **0.038** | **0.039** | **0.151** | **0.725** | **15th day of group 350 HA unit/dose** |

**Table 8: Lymphocyte Blastogenesis of Chickens Vaccinated Inactivated AIV adjuvanted with ISA-70 Adjuvant:**

| Cell proliferation expressed by optical density | | | | days post vaccination |
|---|---|---|---|---|
| control | | AIV- ISA70 | | |
| Without XTT Reagent | With XTT Reagent | Without XTT Reagent | With XTT Reagent | |
| **0.039** | **0.138** | **0.257** | **0.313** | **21th day of group 100HA unit/dose** |
| **0.042** | **0.186** | **0.159** | **0.679** | **21thday of group 256 HA unit/dose** |
| **0.038** | **0.139** | **0.421** | **0.569** | **21th day of group 350 HA unit/dose** |

## DISCUSSION

Influenza A viruses is one of the important pathogens in veterinary and human health around the world. Avian influenza (AI) virus in poultry is unusual in that it can cause a range of disease symptoms from a subclinical infection to being highly virulent with 100% mortality (Suarez, 2008). The emergence in 2004 and continued persistence of highly pathogenic H5N1 influenza A virus in bird populations is justifiably considered a potential pandemic threat (Poland and Sambhara, 2008). The virus has become endemic in many areas of the world and has demonstrated an ability to infect humans through transmission from poultry, thus far with limited human-to-human spread (WHO, 2008). For these reasons, the development of strategies to minimize ries of controlling measure in countries that are currently infected and also a means of reducing the risk of introduction in areas at high risk of infection (Capua and Marangon, 2007).

The progress in vaccination is directed towards the selection of the proper adjuvant that can elaborate high and long lasting immunity. So adjuvants considered one of the important factors in vaccine formulation due to, its influence on the immune response and increase the immune response to vaccines. Adjuvants also can prolong the immune response and stimulate specific components of the immune response either humoral or cell-mediated immunity (SEPPIC, 2002).

The present investigation dealt with the comparative evaluation of 15 experimentally prepared inactivated AI the impact if the virus mutates to acquire efficient human-to-human spread is essential.

Since February 2006 the highly pathogenic (HP) avian influenza H5N1 had emerged as the cause of sever disease and high mortality in chicken on production farm and villages based production of Egypt, by the time the disease had spread all over the country threatening the poultry industry and causing a great hazard to humans. It is known that biosecurity represents the first line of defense against AI, although in certain circumstances strict hygienic measures appear to be in applicable for social and economic conditions and the availability and use of effective vaccine can be valuable tool in controlling outbreaks of AI to maximize the outcome of a se virus (H5N1) vaccines using Montanide ISA 70 oil as adjuvants with different haemagglutination units and different ratios between chicken and Duck strains of Avian influenza.

Propagation of low pathogenic avian influenza virus A\chicken\Egypt\Q1995D\2010 (H5N1) on 9-11 days old SPF ECEs through allantoic cavity revealed highest infectivity titer of virus was 10 log10 EID50/ml and HA titer was 10 log2 HAU/ml as shown in table (1). It was used as the seed virus for production of vaccine as recommended by OIE, 2008. The results of testing cell mediated immunity revealed that the prepared vaccines containing 350HAU/dose induced significant high lymphocyte cells followed by 256HAU/dose followed by 100HAU/dose.

## REFERENCES


**Aguilar, J.C and Rodriguez, E.G (2007):** Vaccines adjuvants, revisited. Vaccine 25:3752 -3762.

**Capua, I. and Marangon. S. (2003):** The use of vaccination as an option for the control of avian influenza. Avian. Pathol. 32(4), 335-343.

**Capua I, and Marangon S (2007):** The use of vaccination to combat multiple introductions of notifiable avian influenza viruses of H5 and H7 subtypes between 2000 and 2006 in Italy. Vaccine 25(27):4987-4995.

**Fouchier, R. A. M.; Munster, V. and Wallensten A.(2005):** Characterization of a novel influenza A virus HA subtype (H16) obtained from black-headed gulls. J. Virol. ; 79(5):2814-2822

**Khafagy, A.K.; S. Attiat; A.A. Sarhan, and R.E. Gough (1992):** Isolation of avian influenza virus (H7N1) from turkeys in Egypt.Proc. 2nd Cong Fac. Vet. Med. Cairo University. pp., 65.

**OIE, (2008):** Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, Office International des Epizooties, Paris, France: World Organization for Animal Health.

**Poland, G. A., and S. Sambhara. (2008):** Vaccines against influenza A (H5N1): evidence of progress.J. Infect. Dis. 198:629-631.

**SEPPIC, (2002):** Montanide ISA Adjuvants for Poultry Vaccines. Seppic Limited Incorporation Company.

**Suarez, D. L., Senne, D. A., Banks, J., Brown, I. H., Essen, S. C., Lee, C., Manvell, R. J.,Mathieu-Benson, C., Moreno, V., Pedersen, J. C., Panigrahy, B., Rojas, H., Spackman, E., andAlexander, D. J. (2004):** Recombination resulting in virulence shift in avian influenza outbreak in Chile.Emerging Infect. Dis. 10, 693–699.

**Suarez, D.L. (2008):** Influenza A virus in Avian influenza 2008 edited by David E.Swayne; 3-22.

**Tiensin,T.; Chaitaweesub,P. and Songserm ,T. (2005):** Highly pathogenic avian influenza H5N1, Thailand. Emerg. Infect. Dis.; 11(11):1664-1672.

**Van der Goot, J.A.; Koch,G. and de Jong, M.C.M.,(2005):** Quantification of the effect of vaccination on transmission of avian influenza (H7N7) in chickens. Proc Natl. Acad. Sci. 13; 102(50):18141-18146.

**Voyles, B.A.(2002): Orthomyxoviruses. In:** The biology of viruses 2nd ed. New York, NY: McGraw-Hill, 147.

**Webster, R.G.; Peiris, M. and Chen H. l. (2006):** H5N1 outbreaks and enzootic influenza. Emerg. Infec. Dis.;12 (1):3-8.

**WHO (World Health Organization) (2006):** Avian influenza: situation (birds) in Nigeria. Feb 8.

**WHO (World Health Organization) (2008):** H5N1 avian influenza timeline of major events. World Health Organization,Geneva,Switzerland. Available at: http://www.who.int/csr/disease/avian_influenza/en