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## **Immunogenicity and Efficacy of Locally Prepared Montanide-oil Based H5N2 AI Vaccine Containing Flagellin As Immune Enhancer**

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### **ABSTRACT**

Objective of the present study was to investigate the efficacy of different vaccine preparations associating inactivated *Avian influenza* (H5N2) antigen, Montanide ISA 70 as an adjuvant and flagellin as an immune-enhancer. Their Immunoenhancing effect in chickens after single vaccination dose (0.5 mL) of the prepared vaccines was assessed. Measurement of cell mediated immunity by lymphocyte blastogenesis at 3rd, 7th, 10th, 14th, 21st and 28th was estimated also phagocytic activity was estimated 3rd, 7th, 10th, and 14th days post vaccination. While humoral immune response, based on Haemagglutinin inhibition test was traced 36 weeks post vaccination. The results revealed that vaccination with the inactivated *Avian influenza* (H5N2) vaccine in combination with Montanide oil ISA 70 provides robust immunity with longer duration and the addition of flagellin as immune-enhancer ensures a significant higher antibodies titer and better cellular immune response than those vaccines not combined with flagellin.

**Key words:** H5N2, montanide ISA 70, flagellin, immune enhancer

### **INTRODUCTION**

*Avian influenza* viruses are highly contagious and variable viruses that are widely spread among wild water and shore birds (Horimoto and Kawaoka, 2001). Infection with AI virus can be a devastating viral disease causing enormous losses in the poultry industry worldwide (Capua and Alexander, 2004).

*Avian influenza* (AI) is a highly contagious viral disease caused by type *A. influenza* virus which is not only able to infect humans, but also a wide variety of avian species (Park and Glass 2007). Between 6 February 2012 and 5 March, 10 new human cases have been reported from Indonesia “2”, Egypt “4”, Vietnam “1” and Bangladesh “3” (WHO, 2012).

Influenza type A viruses 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).

Influenza A viruses are represented in dozens of antigenic subtypes; 17 Haemagglutinin (HA) and 10 Neuraminidase (NA) subtypes (Tong *et al.*, 2012). 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).

In Africa, H5N1 HPAI infection of domestic birds was reported first in Nigeria in early 2006 and subsequently in Egypt, Niger, Cameroon, Burkina Faso, Sudan, Cote d'Ivoire, and Togo. Of these countries, Egypt has been most severely affected by continuous outbreaks, resulting in severe losses in the poultry industry, with >100 human cases, and 34 human deaths. As of July 2008, Egypt reported outbreaks in nine governorates (Gharbiyah, Minufiyah, Kafr Ash Shaykh, Daqahliyah, Sharqiyah, Minya, Jizah, Suhaj, and Luxor) in commercial and backyard poultry, and poultry in live bird market from 7 February to 14 June 2008. At this time, the national veterinary Service (GOVS) declares H5N1 to be endemic in Egypt; Indonesia is the only other country with endemic H5N1 HPAI (Kim *et al.*, 2010).

To control and in an attempt to eradicate the H5N1 HPAI viruses, the Egyptian agriculture authorities have used vaccination, raising biosecurity and used quarantine measures on poultry farms (WHO, 2010; OIE, 2008).

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).

Flagellin; the major structural component of bacterial flagella (Lowy and Hanson, 1964) and the ligand for Toll-Like Receptor 5 (TLR5) (Hayashi *et al.*, 2001). Flagellin has long been known to possess *in vivo* adjuvant activity (Waldmann and Munro, 1975). Its adjuvant activity is believed to result from its ability to promote dendritic cell maturation (Pino *et al.*, 2005) and consequently facilitate antigen specific T cell proliferation (McSorley *et al.*, 2002).

The development of an adjuvanted influenza vaccine using a TLR ligand such as flagellin to induce robust and broad immune responses is a very attractive idea, particularly for vaccines administered intranasally because of the presence of TLR5 in the mucosal surfaces of the respiratory compartment (Skountzou *et al.*, 2010).

The present study was designed to spot the light on the immunogenicity of locally prepared inactivated *Avian influenza* virus (H5N2) vaccine supplemented with different adjuvant in chicken.

## **MATERIALS AND METHODS**

**Chicks and ECE:** Seventy (70), 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. 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-days of age via allantoic sac route.

**Antigen, adjuvants and vaccines:** The low pathogenic *Avian influenza virus A\turkey\CA\209092\02* (H5N2) was propagated on 72 SPF-ECEs through allantoic cavity for antigen stock preparation. The antigen where inactivated by 0.01 m Binary Ethylenimine where the inactivation process was carried out according to King (1991). Two vaccine batches were prepared as water in oil emulsion (W/O) using Montanide ISA70 V obtained from SEPPIC, Cosmetics, Pharmacy Division, and Paris, France. The 1st batch used the oil at a ratio of 3/7 (v/v) aqueous/oil ratio where the 2nd prepared in the same manner except that flagellin which was obtained kindly from Prof. Dr. Saleh M. El Ayouby, Veterinary Serum and Vaccine Research Institute, was mixed and added during the preparation of the final vaccine. Manufacturing process was carried out according to the standard protocol of SEPPIC and manufacture instruction.

**Experimental design:** Chicks were randomly divided into 3 groups: group 1 and 2 contained 25 chicks each, group 3 contained 20 chicks. Chicks of the 1st group were immunized via the intramuscular route in the thigh region with 0.5 mL of the vaccine prepared with ISA 70 V as an adjuvant where those of the 2nd group received the vaccines prepared with the ISA 70 V added to it Flagellin 0.05 mL. The 3rd group chicks were left unvaccinated to serve as a control group.

**Sampling and tests:** At 3rd, 7th, 10th, 14th, 21st and 28th blood samples were collected for measurement of cell mediated immunity by lymphocyte blastogenesis also, phagocytic activity was estimated 3rd, 7th, 10th, and 14th days post vaccination according to Hussien (1989).

Serum samples were collected up to 36 weeks post vaccination and tested by Haemagglutination Inhibition (HI) Test according to standard procedures.

**Statistical analysis:** Cellular immune responses were analyzed statistically using Fischer Exact probability test at  $p < 0.05$  while humoral immune responses were statistically analyzed using Duncan Multiple Range Test at  $p < 0.05$  (SPSS, 2006).

## RESULTS AND DISCUSSION

It was known that the use of strongly adjuvanted vaccines in poultry could partially overcome the need for the accurate matching of vaccine strain in order to confer satisfactory cross-protection and to increase vaccine efficacy (Cattoli *et al.*, 2011).

Our results showed significant increases ( $p < 0.05$ ) in lymphocyte proliferation in the group vaccinated with Inactivated AIV (H5N2) with Montanide ISA 70+ flagellin as adjuvant compared to the group vaccinated with AIV with Montanide ISA70 oil only in the 7th, 10th, 14th, 21st and 28th days post vaccination while in the 3rd day post vaccination there was no significant difference induced between the two groups as shown in Fig. 1.

This results comes in accordance with Cuadros *et al.* (2004) and Pino *et al.* (2005) where they stated that flagellin's adjuvant activity is believed to promote dendritic cell maturation and consequently facilitate antigen specific T cell proliferation.

Both groups were induced significance increases in lymphocyte proliferation compared to non vaccinated control group Fig. 1. The results demonstrated that the oily prepared vaccines greatly

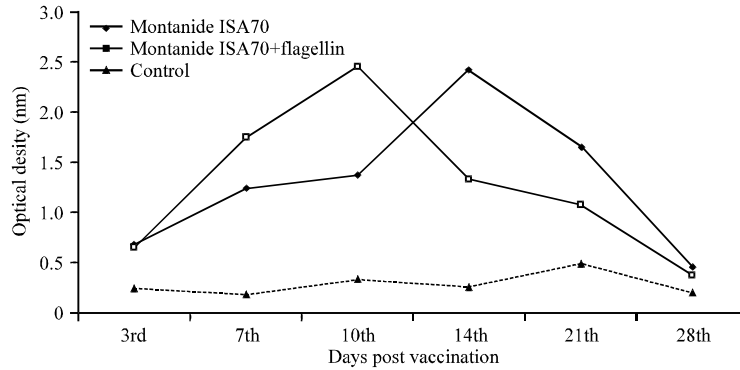


Fig. 1: Evaluation of cellular immune response for inactivated AIV (H5N2) with montanide ISA70 oil adjuvant vaccine compared with immune response of chicks vaccinated with montanide ISA 70+flagellin adjuvant vaccine

Table 1: Phagocytic activities for chicks vaccinated with inactivated AIV (H5N2) montanide ISA70 oil adjuvant vaccine compared to immune response of chicks vaccinated with ISA-70+flagellin adjuvants using *Candida Albicans*

Prepared vaccine	Day 3rd	Day 7th	Day 10th	Day 14th
<b>Phagocytic (%)</b>				
Montanide ISA 70	80.00 <sup>c</sup>	86.70 <sup>b</sup>	88.90 <sup>b</sup>	86.80 <sup>c</sup>
Montanide ISA 70+flagellin	66.70 <sup>b</sup>	85.70 <sup>b</sup>	90.00 <sup>b</sup>	68.80 <sup>b</sup>
Control	2.40 <sup>a</sup>	5.20 <sup>a</sup>	7.60 <sup>a</sup>	5.40 <sup>a</sup>
<b>Phagocytic index</b>				
Montanide ISA 70	0.50 <sup>b</sup>	0.85 <sup>b</sup>	0.81 <sup>b</sup>	0.89 <sup>b</sup>
Montanide ISA 70+flagellin	0.75 <sup>c</sup>	0.83 <sup>b</sup>	0.92 <sup>c</sup>	0.72 <sup>c</sup>
Control	0.02 <sup>a</sup>	0.04 <sup>a</sup>	0.06 <sup>a</sup>	0.04 <sup>a</sup>

a, b, c insignificant difference between similar litters using fisher exact probability test at  $p < 0.05$

stimulated the cellular immune response and similar observation was recorded by others (Abd El-Moneam, 2011) who found that chicken vaccinated with inactivated oil adjuvant AI vaccine induced higher cellular immune response and delayed hypersensitivity test compared with those vaccinated with aluminum hydroxide gel (Cox and Coulter, 1997; O'Hagan *et al.*, 1997).

Phagocytic % and phagocytic index significantly increased with AIV-Montanide ISA 70 and with AIV Montanide ISA 70+flagellin compared to control one Table 1. The phagocytic % and phagocytic index were significantly different between both groups in the 3rd, 10th and 14th days post vaccination as shown in Table 1. The potential effect of flagellin on the magnitude displayed on the activation of cellular immune response of the vaccinated chicks, moreover, the results of macrophage activity test expressed by phagocytic index were supportive to the lymphocyte proliferation results. Collectively the results comes in agreement with those obtained by Tsujimoto *et al.* (2005) who illustrated that both phagocytic percentage and index were significantly increased after vaccination with flagellin. The presence of Toll-like receptor 5 (TLR5) on different cell types including epithelial cells, dendritic cells and macrophages where flagellin binds to this receptor and activate it may be the definite cause of the observed increase in the cellular immunity.

According to De Cassan *et al.* (2011), a central goal in Vaccinology is the induction of high and sustained Ab responses that matches the progress in vaccination which is directed towards the

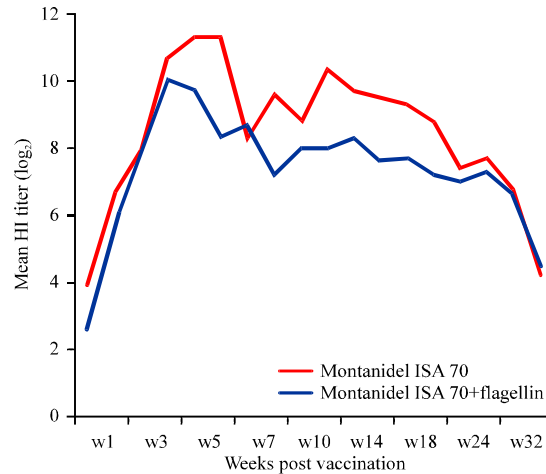


Fig. 2: Humoral immune response for chicks vaccinated with inactivated AIV (H5N2) with montanide ISA70 oil adjuvant vaccine and with montanide ISA 70+flagellin as measured by haemagglutination inhibition test

selection of the proper adjuvant which can elaborate high and long lasting immunity, Liu *et al.* (2011) concluded that an effective vaccine needs not only good antigens but also preferable adjuvant to enhance the immunogenicity of antigen so, the adjuvant was used to enhance humoral and cellular immune responses.

HI serological titers in experimentally vaccinated chicks using AIV-ISA 70 (group 1) and AIV-ISA 70+flagellin (group 2) are presented in Fig. 2. The results showed clearly that, a maximum titers  $10 \log_2$  and  $11.3 \log_2$  were recorded 4th and 5th week post vaccination for both groups, respectively. But the differences between two groups were not statistically significant ( $p > 0.05$ ) in the 4th week post vaccination whereas they were statistically significant in the 5th week post vaccination. Moreover, it's obvious that the HI titers recorded for group 2 were almost higher than those of group 1 along the entire period of the experiment which reflects the role of the flagellin on improving the humoral immune response. Several studies previously reported that flagellin is a potent activator of a broad range of cell types, promotes cytokine production by a range of innate cell types, trigger a generalized recruitment of T and B lymphocytes to secondary lymphoid sites, and activate TLR5+CD11c+cells and Tlymphocytes in a manner that is distinct from cognate (Lee *et al.*, 2006; Mizel and Bates, 2010).

Finally, it could be concluded that the sustained and long standing humoral immune responses were obtained by using the Montanide oil ISA 70 vaccine and Flagellin is recommended as immune-enhancer that can be used with inactivated oil adjuvanted *Avian influenza* vaccine to provoke robust and long immunity.

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