Biochemical and immunogenicity studies on Hemagglutinin protein rescued from H5N1 avian influenza virus like particles

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Abstract: H5N1 subtype of avian influenza (AI) viruses have attracted the current global attention due to its adverse effect on public health, as well as the high economic losses in poultry industry. In Egypt, AI was reported in 2006 and confirmed to be Highly Pathogenic Avian Influenza (HPAI) of subtype H5N1. Egypt was declared endemic country for H5N1 HPAI in July 2008. The weaknesses in the current immunization program in poultry in Egypt require finding a new approach to limit the spread of infection in poultry and increase the maintenance of public health safety. Current study aim was to prepare and characterize HA protein within Virus-like Particles (VLP) and evaluate its expression in the VLP and the ability to stimulate the immune system of the chickens. The preparation of VLP was carried out by optimization of the codon bias of the selected strain and expression of the hemagglutinin (HA), Neuraminidase (N) and Matrix (M1) proteins using Baculovirus expression system. VLPs were characterized using hemagglutination assay, SDS-PAGE, western blot and Immunofluorescense assay. Immunization of the specific pathogen-free (SPF) chickens using the prepared H5N1 VLP demonstrated seroconversion using hemagglutination inhibition (HI) test as a tool for evaluation of the humoral immunity. Thus, VLP has the potential to be developed into an alternative vaccine candidate for the prevention of influenza virus infection in the poultry.
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1-Introduction

Avian influenza (AI) viruses are causing disease in many species including humans. The highly pathogenic avian influenza (HPAI) viruses of H5N1 subtype cause human deaths, adverse effect on public health and high economic losses for agriculture as mortality rate can reach 100% within 48 hr in poultry ***(Horimoto and Kawaoka, 2001)***.

In Egypt, AI was reported on 17 February 2006. The virus was isolated and confirmed to be HPAI genotype due to the presence of multiple basic amino acids in the viral surface Hemagglutinin (HA) cleavage site and the sequences was RRRKKR*GLF. This virus was similar to the Qinghai strain, which firstly detected in western China in 2005 ***(Aly et al., 2006 a).*** The disease has become enzootic in poultry throughout Egypt and still circulates in the poultry population ***(Aly et al., 2006 a and b).***

Human infections with AI (H5N1) was first identified in Egypt in March and April 2006 and was mainly due to exposure to/or handling sick household poultry and all cases were treated with oseltamivir using WHO guidelines ***(Earhart et al., 2009).*** From the beginning of AI outbreak in 2006, until the 2nd of October 2014, Egypt reported 177 confirmed human cases of the H5N1 virus infection, from which 63 have been fatal. Egypt is ranking the second after Indonesia in the number of infections and third after Indonesia and Vietnam in the number of fatal cases ***(WHO, 2014).***

Egypt pursued multiple strategies in controlling of the avian influenza disease, which achieved only limited success. Recent assessment study showed the inefficiency of the current vaccination programs ***(Peyre et al., 2009)***. Vaccination is the most effective strategy for prevention of influenza infections by inducing a virus neutralizing immune response to influenza virus (**Pushko et al., 2011).**

VLP platform vaccines have received significant attention for their potential promise in developing effective and safe vaccines against viral pathogens **(Kang et al., 2009).** Influenza VLPs resemble intact virions in structure and morphology, and contain functionally active and immunologically relevant structural proteins. The viral glycoproteins on VLPs are in a native conformation and unmodified by fixatives or chemicals for inactivation **(Bright et al, 2007 and Kang et al., 2009).** So this study aimed

to prepare and characterize HA protein within VLPs using Baculovirus expression system and to evaluate its ability to express in Sf9 cells and to stimulate the immune system of the chickens using Hemagglutination Inhibition test.

2-Materials and methods

2.1-Sampling, detection and propagation of the virus

The Egyptian AI Virus (AIV), A/chicken/Egypt /121/2012(H5N1) was collected from commercial clinically diseased chicken farm during AIV surveillance, and tested for AIV using real-time reverse transcription-polymerase chain reaction (RT-PCR). The viral RNA extraction was performed using a QiaAmp viral RNA mini kit (Qiagen, Germany) according to the manufacturer's instructions and the Real-time RT-PCR was performed using a one-step method for avian influenza A virus matrix (M), H5 and N1 genes, using specific primers and probes **(Spackman, et al., 2002, Slomka, et al., 2007 and WHO. 2007)** respectively. The pooled swab samples were inoculated via allantoic route in SPF eggs for propagation and were confirmed for the presence of H5N1 virus after isolation by Real-time RT-PCR.

2.2-Molecular characterization of the selected isolate.

The sequencing of the H5, N1, and M1 genes of A/chicken/Egypt/ 121 /2012 (H5N1) were achieved after amplification of the full length of these genes using Qiagen one step RT PCR (Qiagen, Germany) by the specific primers. The nucleotide sequence of the three genes were determined by BigDye Terminator Kit (version 3.1; Applied Biosystems, Foster City, CA) using the same specific primers of the amplification and internal primers for the full-length coding gene sequencing.

2.3 Preparation of VLP.

The full-length H5, N1, and M1 genes were codon-optimized for high-level expression in Spodoptera frugiperda (Sf9) cells (ATCC, Manassas, VA) and synthesized biochemically. Recombinant baculovirus (rBV) expressing H5, N1, and M1 genes were constructed using a Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA)**.** Each full-length of H5, N1 and M1 genes were transferred from pUC 57 into pFastBac1 transfer vector using BamHI and Hind III restriction enzymes and each gene was placed within its own transcriptional cassette that included an AcMNPV polyhedrin promoter upstream from each gene. The three genes were combined within a single pFastBac1 transfer vector by cloning using HpaI, PvuI and SnaBI restriction enzymes to form recombinant H5 N1M1 pFastBac1. After that, recombinant bacmids were produced by site-specific transposition following the transformation of the combined transfer plasmid containing H5, N1, and M1 genes into E. coli DH10Bac competent cells, which contained the AcMNPV baculovirus genome (Invitrogen),

The transfection of the recombinant bacmid DNA was carried out into the Sf9 insect cells seeded in 6-well plates at 2×10^6cells/2ml /well using Fugene® reagent for 72 h. at 27 C^O. Recombinant baculoviruses were recovered from the culture medium and tested by hemagglutination test. For plaque purification, the Sf9 cells seeded in 12 well plate of 0.8×10^6 cells /2ml/well were infected by serial dilution of the transfection harvest and incubated for 10 days to form plaques. The agarose plugs were extracted and incubated in SF-900 II SFM (Invitrogen) to recover virus from plaques. Approximately 0.5 ml of the plaque eluate was added into 20 ml of high-viability insect cells SF9 at $1X10^6$ / ml concentration and incubated at 27 C° on shaker incubator for 3 days that resulted in the P1 baculovirus. The P1 was tittered by plaque assay at 0.8×10^6 cells / 2ml/well. For protein expression, Sf9 cells were infected with recombinant baculoviruses for 72 h at a cell density of 2×10^6 cells/ml at a multiplicity of infection (MOI) = 3. Culture supernatants were harvested and clarified by centrifugation for 3000 rpm/15 min.at 4 C^O, the supernatant was filtrated using sterile 0.2 µm filter. VLPs were pelleted for 2 h at 28000 rpm 4 C^O by ultracentrifugation of the filtrate. The pellets were resuspended in phosphate buffered saline (PBS), pH7.4.

2.4. Characterization of the expression of the VLPs.

The expression of the VLP was subjected to the confirmation and characterization at different steps during preparation by testing the ability to agglutinate red blood cells (RBC). Serial 2-fold dilutions of VLPs were prepared in 96-well microtiter plates, followed by the addition of 1% turkey RBC (Lampire Biologicals, Pipersville, PA) in PBS. RBCs were allowed to settle for 30 min at room temperature, and the HA titer was determined by visual inspection. Proteins within VLP preparations were separated using 4–12% gradient SDS-PAGE, stained with GelCode Blue stain reagent (Thermo Scientific, Rockford, IL). Western blots of VLPs were done using chicken polyclonal H5 antiserum against avian influenza H5 followed by alkaline phosphatase-conjugated anti-chicken IgG. Immunofluorescense assay (IFA) was done using specific antisera for H5 influenza. For fluorescent staining, 0.3 ml aliquots of infected Sf9 cells were seeded into eight well Nunc LabTek slides. Following 72 h incubation at 27 C^0, Sf9 cells were fixed with cold acetone, and IFA was carried out as described elsewhere **(Pushko et al.,**

2001). Antigen expressing cells were visualized using FITC-conjugated goat species specific IgG (H+L) (KPL, Gaithersburg, MD).

2.5. Evaluation of the immunogenicity of the VLPs in chickens.

In the experiment 10 SPF white leghorn chicks at 4 week of age were immunized one time subcutaneously (S/C) with VLPs prepared from Egyptian stain A/chicken/Egypt/121/2012 (H5N1). The VLPs were diluted in PBS buffer and adjusted to 10^9 HA unit.Another 10 SPF white leghorn chicks at 4 week of age were considered negative control group and received PBS in place of VLPs. The blood samples were collected from wing vein before the administration of VLPs and after 3 weeks of the administration. The determination of the humoral immunity induced by VLPs was carried out by hemagglutination inhibition assay (HAI) by detecting antibodies using 4 hemagglutination units of the prepared VLPs, which were mixed with serial dilutions of antibodies from VLP-immunized SPF chickens prior to addition of 1% chicken blood cells according to the standard protocol **(OIE, 2009).**

3-Results

3.1. Molecular characterization of the collected isolate.

The collected sample was typed as Influenza A and subtyped as H5N1 using Real-time RT-PCR for M gene and HA and NA genes respectively, by sequencing it was characterized as H5N1 strain of 2.2.1 subclade. The phylogenetic analysis using NCBI BLAST software has shown that HA protein derived from our isolate A/chicken/Egypt/121/2012 (H5N1) was similar to other H5N1 strains from the Middle East region including A/duck/ Egypt/ 11762s /2011,A/quail/Egypt/1171SG/2011,A/harrier/Israel/ 531/2011, as well as to human isolate A/Egypt / N02137/2012.

3.2. Optimization of the nucleotides sequence of the selected strain.

The sequence was optimized for high expression level in insect cells. The similarity index between the original and the optimized nucleotide and amino acid sequences is shown in table (1).

Confirmation of the proper construct of combined pFastBac1-H5N1M1 using restriction enzymes.

The proper construct was confirmed by digestion screening using Tth111I and PvuII restriction enzyme as shown in Figure (1).The figure shows the specific weights of the digested pFastBac1-H5N1M1 fragments, Figure (1a), by using Tth111I restriction enzyme producing 5158 bp, 2211bp, 1719 bp specific fragments. Figure (1b), by using PvuII restriction enzyme producing 7075bp, 1673bp, 342bp specific fragments. The diagram (figure1c) illustrates the arrangement of the hemagglutinin (HA), Neuraminidase (NA)and Matrix (M1) proteins within the Baculovirus Bacmid showing that each gene within its own expression cassette

Table (1): Similarity index between the original viral sequence (nucleotides and amino acids) and the optimized sequence for the essential genes for the VLP construction (H5, N1 and M1)

Gene	Nucleotides similarity index	Amino acids similarity index
H5	75%	100%
N1	74%	100%
M1	74%	100%

3.3- Characterization of the expressed proteins within VLP

By using 4–12% gradient SDS-PAGE, stained with GelCode Blue stain reagent (Pierce, Rockford, IL), the proteins within VLP preparations were separated as shown in the figure (2a), HA protein of about 64 KD, and confirmed by western blotting giving the specific band as shown in the figure (2b) using the polyclonal antiserum against avian influenza H5 subtype. Furthermore, the expression of HA protein was confirmed by immunofluorescence assay (IFA) using the specific antibodies as shown in figure (2c).

Hemagglutination test for testing the expression of functional HA protein

Hemagglutinin protein has a biological function of agglutination of the red blood cells. In this study the HA test was used for detecting the proper expression of the HA protein in different steps during the preparation of the VLPs as shown in figure 3. The HA result indicate the expression of functional HA protein

3.4. Evaluation of the immunogenicity

In order to test immunogenicity of the VLPs, SPF chickens were inoculated with experimental VLP vaccine as described in Materials and Methods. After immunization of the SPF chickens by the prepared VLPs blood samples were collected in 3 weeks and HAI assay was carried out for the serum in comparison to the negative group as shown in table 2.

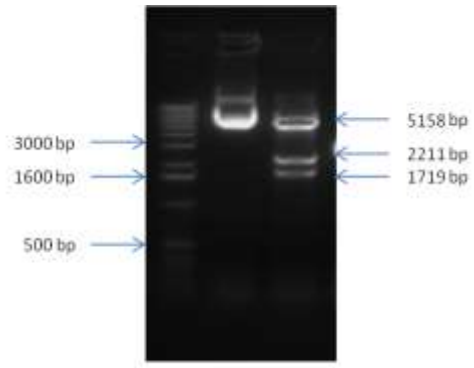

Figure 1a

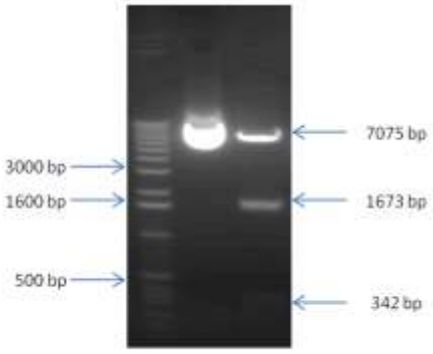

Figure 1b

Figure 1- Electrophoresis of the digested pFastBac1-H5N1M1 construct.
The figure 1a illustrates the digestion of the pFastBac1-H5N1M1 with Tth111I while figure 1b with PvuII restriction enzymes.The ladder fragments are 500 bp, 800bp, 1600 bp, 2000 bp, 3000 bp, 4000 bp, 5000 bp, 6000 bp.... etc, the both figures shows the undigested recombinant pFastBac1-H5N1M1 as a control.

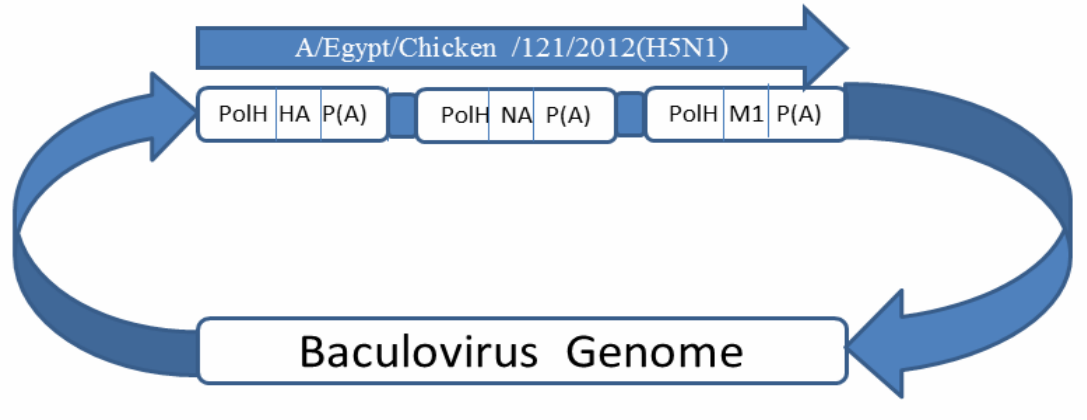

Figure 1c
This diagram shows the arrangement of the hemagglutinin (HA), Neuraminidase (NA) and Matrix (M1) proteins, within the Baculovirus Bacmid.(PolH, polyhedrin promoter; P(A), polyadenilation signal; and influenza A/ chicken/ Egypt/121/2012(H5N1) genes, HA, hemagglutinin; NA, neuraminidase; M1, matrix protein).

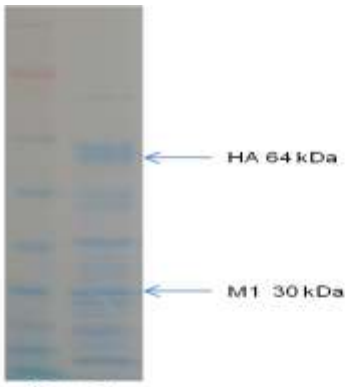

Figure 2a

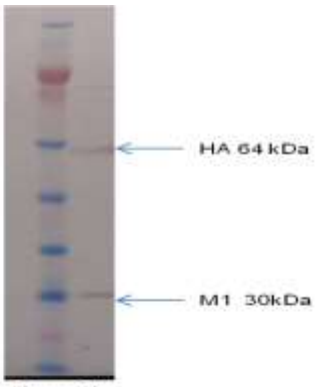

Figure 2b

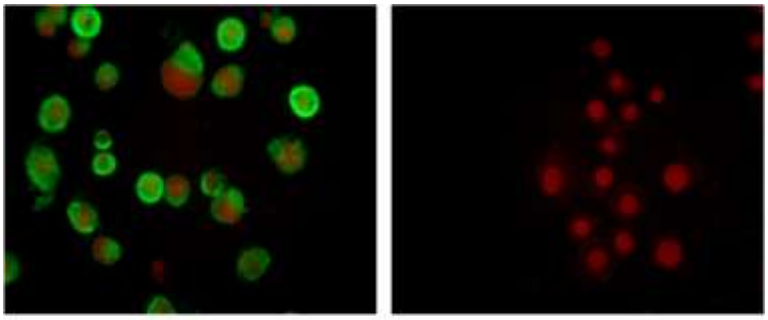

Figure 2c

Figure 2: SDS-PAGE, western blot and Immunofluorescence assay (IFA) for characterization of VLP expressed proteins. The figure (2a) shows the separation of VLP expressed proteins using 4–12% gradient SDS-PAGE and SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) showing the full-length HA protein of about 64 kDa and M1 of about 30 kDa, NA not appear due to it is expressed in low amount in comparison to other influenza virus proteins, while in figure (2b) using western blot shows the specific band of the whole HA. In fig (2c) another characterization of the HA protein by IFA using specific antibodies producing positive reaction as the cells infected by the recombinant baculovirus expressing HA(1) in combarison with uninfected SF9 cells (2).

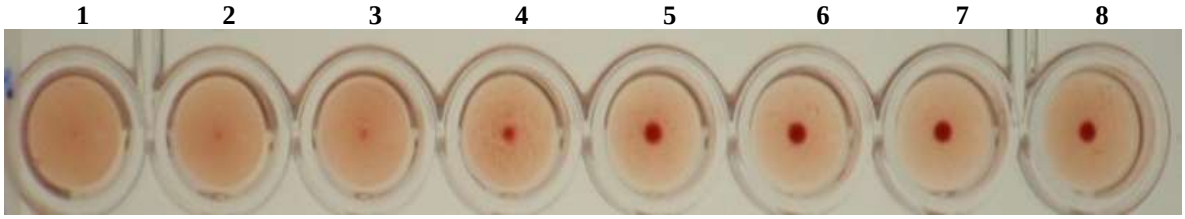

Figure 3a

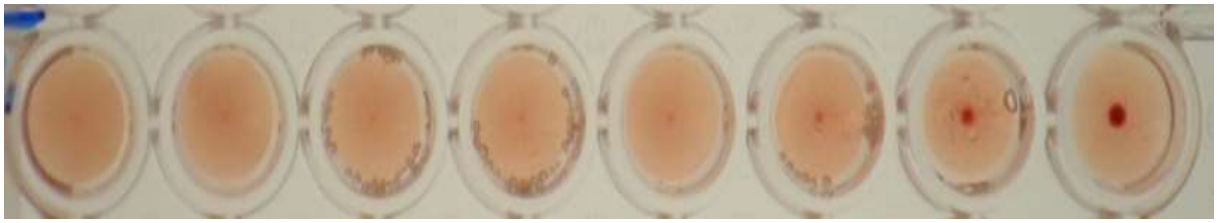

Figure 3b

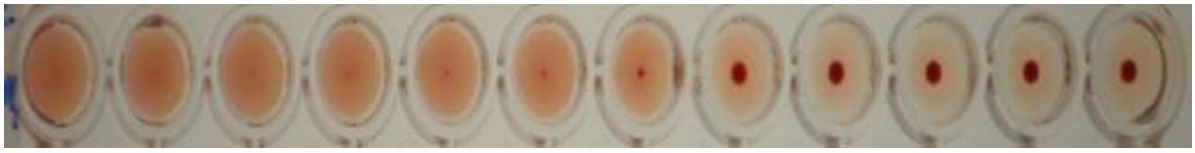

Figure 3c

Figure 3: Hemagglutination test of VLP at different steps of preparation.

The figure (3a) shows HA activity of the transfection harvest at 2^3 titer using 1% turkey RBCs and the start dilution was 1: 1.The figure (3b) shows HA activity of the Passage 1 (P1) harvest at 2^6 titer using 1% turkey RBCs and the start dilution was 1: 1.The figure (3c) shows HA activity of the expressed VLP harvest at 2^{12} titer using 1% turkey RBCs and the start dilution was 1: 128

Table 2. HI titer for detecting Antibodies against H5N1 VLP.

Bird No.	HI titer after 3 week of immunization (HA unit) log 2	HI titer of Negative control (HA unit) log 2
1	2^4	ND
2	2^5	ND
3	2^2	ND
4	2^4	ND
5	2^5	ND
6	2^4	ND
7	2^5	ND
8	2^4	ND
9	2^5	ND
10	2^4	ND
Average	$2^{4.2}$	

The result confirmed elicitation of the humoral antibodies after H5N1 VLP immunization in comparison to the negative group. These data confirm immunogenicity of experimental H5N1 VLP as a potential veterinary vaccine for poultry.

4-Discussion

Avian influenza virus (H5N1) spread rapidly in Egypt infecting poultry species in either commercial or backyard poultry sectors raised in Egypt, causing high economic losses as mortality that can reach 100 %. Furthermore, frequent human infections were detected suggesting the possibility of H5N1 AI outbreak in people. Many studies evaluate the evolution of the AI virus (H5N1) in Egypt revealing that there are 2 major subclades for the Egyptian strains; classic 2.2.1 and variant 2.2.1.1 **(Ibrahim et al., 2013).** Recently the classic strain became more dominant than the variant one in the Egyptian field according to the published data in GeneBank, but these recent classical strains differ as they evolved from the original classical strains which were isolated in 2006. By comparison of the classical strain in 2006 (EU372944) and the classical strain in 2012 (JQ858483) it was found 97% similarity for both Nucleotide and Amino Acid sequences giving indication that these mutations may be related to the immune pressure. Inactivated H5N1 and H5N2 whole virus vaccines with different seed strains were applied in 2006 along with other control measures **(Peyre et al., 2009)**. The virus still continued to circulate in the vaccinated commercial and backyard poultry due to vaccine inefficiency that may be related to defects in vaccine quality or other factors related to storage, handling and administration of the vaccine **(Hafez et al., 2010)**. These indications of the weakness of the current vaccination strategies in Egypt suggested the need for finding a new approach to control the spread of infection in poultry and to improve public health safety. The selection of the strain in this study was from the current circulating viruses of subclade 2.2.1 accession No. (JQ858483) which was optimized (table 1) for the high expression in insect Sf9 cells as revealed in HA test of the transfection and passage 1 harvest which were 2^3, 2^6 HA unit respectively, as shown in fig (3a) and fig (3b).The essential genes (HA,NA,M1) for preparation of VLP were combined in arrangement as illustrated in fig (1c). This gene arrangement may differ than other construct as published before **(Pushko et al., 2005).** However**,** the difference in the arrangement of the genes is not expected to affect the expression as each gene is expressed from its own expression cassette. The combined pFastBac1+H5N1M1 was confirmed by restriction enzyme digestion screening using different restriction enzymes as shown in fig (1a) and (1b). The expression of the HA protein as it is the most immunogenic molecule on the surface of the influenza virus and the antibodies were elicited toward it, was characterized by SDS-PAGE giving specific band at approximately 64 kDa, Western blot produced positive reaction as shown in fig (2a) and fig (2b) respectively. Immunofluorescence assay (IFA) of Sf9 cells infected with recombinant baculovirus expressing HA used for characterization of HA expression as in fig (2c). The integrity of influenza VLPs maintaining hemagglutination activity seems to be a critical factor in inducing functional antibodies that are protective **(Quan et al., 2007**), in this study HA titer of the expressed HA protein within VLPs was 2^{12} as shown in figure (3c).The immune system has multiple mechanisms to vigorously respond to virus particles which may be exploited by VLP-based vaccines **(Grgacic and Anderson, 2006 and Spohn and Bachmann, 2008)**. In practical terms, the fact that VLPs mimic the structure of virus particles usually means that lower doses of antigen relative to monomeric antigen vaccines are sufficient to elicit a similar protective response.This consideration is particularly significant in the case of veterinary vaccines, where the cost of a vaccine must be weighed against the value of the vaccinated animal **(Brun et al., 2011)**. Historically, the HAI assay is the most widely used serological assay for monitoring influenza immunity and is the accepted standard for measuring functional influenza-specific serum antibodies to the hemagglutinin following vaccination **(Bright et al., 2008).** Two doses of VLPs containing 3µg or 600 ng HA provided broadened protective immune responses including humoral and cell-mediated immunity, which was found to be protective against challenge infections with reassortant homologous (A/Indonesia /05/2005) or a heterologous virus, A/Vietnam/ 1203 /2004(Viet04)clade 1**(Bright et al., 2008 and Crevar and Ross, 2008.).** In this study, the ability of our

constructed HA protein from recent Egyptian strain A/chicken/Egypt/121/2012 (H5N1) within VLP molecules to induce the immune system was measured using HAI assay after one dose of PBS based VLP subcutaneously of 4 week of age of SPF chicks. As shown in table (2), the average of the HAI titer was $2^{4.2}$ these mean that this construct can trigger the immune system suggesting the feasibility for developing immunogenic VLP vaccines in poultry. As presented before Influenza VLPs have been shown to be a promising vaccine candidate because of their robust immunogenicity and easy elicitation of protective neutralizing antibodies due to the presence of conformational epitopes on VLPs, and their advantageous safety profiles **(Pushko, et al., 2005, Noad et al., 2003, Pumpens et al., 2003 and Schiller and Hidesheim, 2000).** From this study, we may conclude that the VLP construct is immunogenic, furthermore, the safety issues in the VLP producing procedure. VLP vaccines have also potential immunologic advantages over split or purified viral protein vaccines mainly due to VLPs contain multiple copies of antigens presented in an organized array, thus allowing activation of the innate immune system and the size of VLPs allows efficient uptake and processing by professional antigen presenting cells, i.e. dendritic cells and macrophages, for presentation to both T and B lymphocytes to stimulate humoral and adaptive immune responses **(Kang et al., 2009, Buonaguro et al., 2006, Da Silva et al., 2001, Lenz et al., 2003, Moron et al., 2002, Moron et al., 2003 and Sailaja et al., 2007).** Thus, VLP has the potential to be developed into an alternative vaccine candidate for the prevention of influenza virus infection in the poultry.

Acknowledgments

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