Protective efficacy of H5 based DNA vaccine prepared from Egyptian H5N1 avian influenza virus

Mady W. H. [1*], Arafa A.[1], Hussein A. S. [2], Aly M. M. [1] and Madbouly H. M. [2]

[1]National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, P.O. Box 264-Dokki, Giza-12618, Egypt
[2]Virology Department Faculty of Veterinary Medicine, Beni-Suif University, Egypt
*Corresponding Author: Mady W. H.
National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, P.O. Box 264-Dokki, Giza-12618, Egypt, Tel: 202 33380121, Fax: 202 33370957, Mobile: 01096105189 – 01147276255
Email: wesammady83@hotmail.com, wesammady@gmail.com

Abstract: The incursion of highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 into Egypt in 2005/2006 caused severe economic losses in the commercial and backyard sectors of poultry production. Since the DNA vaccine seemed to be a promising novel approach for vaccination against influenza A virus, the goal of this study was to prepare HA1 based DNA vaccine against the H5N1 avian influenza viruses circulating in Egypt. The HA1 gene from Egyptian virus A/chicken/Egypt/1055/2010(H5N1) was extracted and cloned into PCIneo mammalian expression vector. The invitro expression of recombinant H5-plasmid DNA was confirmed in 293T human embryonic kidney (HEK) cell line and in Vero cell line by SDS-PAGE and by detection of mRNA using RT-PCR. The immunological response was investigated by intramuscular immunization of SPF chickens with PCIneo-H5 lipofectamine adjuvant vaccine. The immunological analysis showed that PCIneo-HA1 vaccine induced both humoral and cell mediated immune response in chickens. The protection of H5-DNA vaccine was evaluated by challenge of 3 weeks old SPF chickens vaccinated with PCIneo-H5 DNA vaccine using one dose of conc. 5µg/dose by direct intramuscular injection in thigh muscle and compared with protection afforded by H5N2 AI vaccine. The result showed that the H5 DNA vaccine protected the chickens from the Egyptian field H5N1 virus better than the H5N2 inactivated vaccine (60% versus 40% respectively). This is the first paper describing the development and primary evaluation for DNA vaccine prepared from Egyptian H5N1 virus. Further adjustments of the newly developed H5-DNA vaccine dose and vaccination regimen are required to better protect chickens against the virulent field viruses.
[Mady W. H., Arafa A., Hussein A. S., Aly M. M. and Madbouly H. M. **Protective efficacy of H5 based DNA vaccine prepared from Egyptian H5N1 avian influenza virus.** *J Am Sci* 2013;9(7):215-223]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 26

Keywords: H5 based DNA vaccine, highly pathogenic avian influenza virus H5N1, PCIneo mammalian expression vector, lipofectamine adjuvant, SYBER Green quantitative real time PCR for cell mediated immunity evaluation, protective efficacy of H5 vaccine.

1. Introduction

In Egypt, severe outbreaks due to infections with HPAI H5N1 virus of clade 2.2 in poultry were recorded since February 2006. A nationwide blanket vaccination campaign attempting to reach all poultry species in all holdings was implemented to decrease the overwhelming economic impact of the disease on poultry industry and to mitigate the risk of trans-species transmission to the human population **(Abdelwhab et al., 2011).** However, already in 2007 virus was detected in 35 vaccinated farms, and after 18 months of mass vaccination, antigenic drift variants, phylogenetically characterized as clade 2.2.1 viruses (designated "variant, A or E"), were detected **[Aly et al., 2010, Arafa et al., 2010, and Balish et al., 2010).** These variant 2.2.1 viruses now widely dominate the epidemiology of HPAI H5N1 among sector 1 and 2 chicken holdings in Egypt **(Abdelwhab et al., 2011).** Phylogenetic analysis of Egyptian strains isolated in 2007 from El-Qanater El-Khiria (backyard) revealed that the Egyptian isolates of 2007 showed some antigenic minor variation from those isolated in 2006 from the same locality and were located in a new subclade **(Madbouly et al., 2012).** Despite the blanket vaccination strategy against HPAI H5N1 in Egypt, continuous circulation of the virus in poultry has increased since late 2007 as a result of the presence of genetic and antigenic distinct variant strains that have escaped during the immune response of vaccinated birds **(Kilany et al., 2011)**.Therefore, a vaccine that could protect chickens from a lethal infection and prevent the spread of the virus is urgently needed.

Currently, whole virus inactivated vaccines containing HA as the main component, are the common vaccines to prevent avian influenza. However, these vaccines require large numbers of specific-pathogen-free embryonated chicken eggs and about 6 months to propagate the viruses **(Lewis,**

2006). On the other hand, this is not an ideal method to produce inactivated vaccine for highly pathogenic strains, as the embryos are killed shortly after propagation and require a high level of biosecurity to handle **(Voeten et al., 1999)**.

The development of cost-effective avian influenza (AI) vaccine is a priority to prevent pandemic flu outbreaks. The DNA-based immunization is a promising strategy to prevent persistent viral infections and diseases. This approach can induce a broad range of immune responses and has been successfully used to provide protective immunity against influenza **(Montgomery et al., 1993 and Donelly et al., 1995)**.

The influenza virus is comprised of 11 proteins, and the HA is no doubt the major target for protective immunity. Antibodies against this surface glycoprotein can provide protection by blocking virus attachment and entry **(Lee et al., 2006).**

Because hemagglutinin (HA) protein is a major viral surface antigen against neutralizing antibodies elicited, recombinant HA was a target as a candidate avian influenza vaccine.Perhaps most important, the HA-DNA vaccine conferred 95% protection against challenge with lethal antigenic variants that differed from the primary antigen by 11-13% (HA1 amino acid sequence homology) **(Kodihalli et al., 1997).**

Since most antigenic and neutralization sites are in the HA1 domain of HA **(Shih et al., 2007)**, using HA1 domain of influenza virus as antigen is of great importance in vaccine development **(Fang-Feng et al., 2009)** specially in countries facing vaccination failure inforce endemic viruses to be adapted like in Egypt.

2. Material & methods

Selection of DNA vaccine strain & virus propagation, titration and isolate confirmation

The selected DNA vaccine strain was A/chicken/Egypt/1055/2010(H5N1) variant H5N1 (NLQP), Gene bank Accession no. HQ198268. The virus was propagated on Confluent monolayer of Madin-Darby Canine Kidney cells (MDCK) & titrated by calculating the TCID50 by using Reed & Meunch method **(Reed and Muench, 1938)**. Plaque assay was used to measure plaque forming unit (PFU) on MDCK.

The propagated virus was confirmed for H5 gene detection by H5 Reverse transcriptase Real time PCR (RRT-PCR), Viral RNA extraction was accomplished using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, Calif., USA) according to the manufacturing instructions as procedures & Real time PCR for H5 subtype was performed with Quantitect probe RT-PCR kit (Qiagen, Inc. Valencia CA) and Stratagen MX3005P machine (Stratagene, USA) by using H5 primers and probe as previously described **(Spackman et al., 2002).**

Preparation of HA plasmid DNA construct

The cDNA synthesis for the extracted RNA was performed using Access Quick RT-PCR (promega, USA) using Uni-12 primer: AGCAAAAGCAGG then HA1 gene was amplified by using Primers containing restriction sites:

FH5-NheI-PCI-neo:
CGTA***gctagc***ATGGAGAAAATAGTGCTTCTTCTT GCAA TATT and RH5-HA1-XhoI-PCI-neo: GCAT***ctcgag***CTATTTCTGAGCCCAGTA GCAAGGACC

HA1 gene was cloned in pGEM® T Easy Vector System I (Promega, USA) & transformed in JM107 E.coli cell (ferments, Canada) using TransformAid™ , The cloned HA1 gene was then cut with NheI & XhoI restriction enzymes 10µ/µl (promega) by double digestion & directionally subcloned into PCI-neo mammalian expression vector 1mg/ml (promega, USA) which contains the cytomegalovirus (CMV) early promoter with T4 DNA ligase 2,000,000 cohesive end units/ml (New England Biolabs). The recombinant plasmid DNA (PCI-neo/HA1) was then transformed in JM107 E.coli cell (ferments, Canada) using TransformAid™ Bacterial Transformation Kit (ferments, Canada), the recombinant plasmid DNA was extracted using Qiagen plasmid maxiprep kit (Qiagen, Valencia, Calif., USA)

The constructed PCIneo-HA1 recombinant plasmid was sequenced for confirmation of proper ligation at both forward & reverse junctions of the construct by using sequence primers H5-F4-817: AGTAATGGAAATTTCATTGCTCCAGAA & H5-R6-395: TTTATTCTGCTCAATAGGTGTT.

The constructed recombinant plasmid DNA (PCI-neo/HA1) were electrically transfected in 293T human embryonic kidney (HEK) cell line suspension of Cell count 1×10^6 cells per transfection reaction by electroporation by using CLB transfection kit (lonza, Germany), & electroporator CLB-Transfection device (lonza) for confirmation of HA1 protein expression which was detected by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) **(Laemmli, 1970)**. The ability of the constructed H5 plasmid DNA vaccine in inducing mRNA expression for H5 was studied using oligo $(dT)_{18}$ primer & RT-PCR following transfection in 293T (HEK) cell line **(Jalilian et al., 2010).**

Evaluation of H5 DNA vaccine immune response

Ten SPF chickens of 3 weeks old were immunized with single dose of 10 µg plasmid DNA mixed with lipofectamine, 0.5 ml by direct intramuscular injection in thigh muscle, another group of 10 SPF chickens of 3 weeks old was used as negative control.

Evaluation of humoral immune response by haemagglutination inhibition (HI) test

Serum from vaccinated birds was tested for HA-specific antibodies with the hemagglutination inhibition test **(OIE, 2009)**.

Evaluation of cell mediated immune response

Phagocytic activity and percentage of chicken peripheral monocyte was determined by using C. albicans according to **(Barry and John, 1988 and Richardson and Smith, 1981).** Blood samples were collected, from each chick by wing vein puncture, in sterile plastic centrifuge tube with heparin (20 IU/ml) for macrophage cells separation (3, 7, 14, 21, 28, 35, 42) days of age for detecting the phagocytic activity of macrophages)

The phagocytic activity was calculated according to the following equations:

Percentage of phagocytosis =

$$\frac{\text{No. of ingesting phagocytes X 100}}{\text{Total No. phagocytes including non-ingesting cells}}$$

Phagocytic index =

$$\frac{\text{Total No. phagocytes with more than 3 blastospores}}{\text{Total No. phagocytes ingesting blastospores}}$$

The lymphocyte proliferation assay was measured by using XTT cell proliferation assay kit (ATCC) cat. no. 30-1011K according to the instruction manual & measuring the absorbance of the assay by ELISA reader. Blood samples were collected from each chick by wing vein puncture with heparin (20 IU/ml) for lymphocyte proliferation assay at (3, 7, 10, 14, 21, 28) days of age.

Interleukin 6 (IL6) analysis, the main Th2 cytokine, by two step SYBER Green quantitative real time PCR (qPCR)

Total RNA was extracted from chicken lymphocytes using TRIzol (Invitrogen, Carlsbad, CA) as described in manual, Total RNA was reverse transcribed by Access quick RT-PCR (promega), cat no. A1702 by using oligodt primer

Amplification and detection of IL6 were carried out using Maxima™ SYBR Green qPCR Master Mix (2X), ROX Solution provided (fermentas,) cat no. #K0259 for 60 reactions of 25 µl. IL6-F5'-CACACAGACAGCCACTCACCTC-3', IL6-R 5'-CTGCCAGTGCCTCTTTGCTG-3', β-actin*-F5'-GTCCTCGGCCACATTGTGA-3' β-actin*-R5'-CTGGAACGGTGAAGGTGACA-3' an internal positive control by using primers according to **(Rafael et al., 2011)**.

Protective efficacy of H5 DNA vaccine

Challenge test was done in 3 weeks old SPF chickens divided into 4 groups, 1st group was vaccinated with HA1 DNA vaccine of conc. 5mg/ml, 0.5 ml per dose by direct intramuscular injection in thigh muscle, the 2nd group vaccinated with volvac H5N2 inactivated vaccine 0.5 ml per dose by S/C injection for comparison, the 3rd group was positive control (challenged not vaccinated), & the 4th group was negative control (not challenged, not vaccinated). Chickens were challenged intranasal with 100 µl 10^6 TCID50 of variant strain H5N1 (A/chicken/Egypt/1055/2010(H5N1) (NLQP). Cloacal swabs were collected at 2nd day post challenge to detect virus shedding by real time H5 RT-PCR as described before.

3. Results

Preparation of HA plasmid DNA construct

The Egyptian virus A/chicken/Egypt/1055/2010(H5N1) was propagated in MDCK with daily microscopic examination; CPE appeared on the infected cell showed cell rounding & vaccules after 48 hours, Darkness & destruction in the cell monolayer & completely detached cell monolayer after 72 hours, the virus was harvested from MDCK infected cell at 3rd day. Virus titration showed that the virus titer was $\mathbf{10^{-10.5}}$ TCID50 & result of plaque assay was $\mathbf{2.85 \times 10^6}$. The propagated virus was confirmed by real time H5 RT-PCR showing crossing threshold value (Ct) at 19.

The HA1 gene were amplified by RT & PCR using primers containing the restriction site showing the expected size of HA1 which is 1016 bp. screening of positive colonies of cloned HA1 gene in pGEM® T Easy Vector showed the expected weight which was 1016 bp in agarose gel electrophoresis. The recombinant plasmid containing the HA1 gene showed the expected weight which was 4031 bp in agarose gel electrophoresis, as it represent the pGEM® T Easy vector system I cloning vector (3015 bp) & the HA1 gene (1016 bp) giving band at 4031 bp.

Both the cloned HA1 gene & the PCI-neo expression vector were digested by NheI & XhoI restriction enzymes for directional subcloning, the free digested HA1 gene showed the expected weight which was 1016 bp, the empty pGEM® T Easy vector system I cloning vector showed 3015 bp, & the PCI-neo expression vector showed 5472 bp in agarose gel electrophoresis. Screening of positive colonies of directionally ligated & subcloned HA1 gene in PCI-neo mammalian expression vector showed the expected weight which was 1016 bp in agarose gel electrophoresis. The recombinant plasmid containing the HA1 gene showed the expected weight which was 6488 bp in agarose gel electrophoresis with GeneRuler GeneRuler™ 1 kb Plus DNA Ladder, fermentas, as the weight of the PCI-neo mammalian expression vector (5472 bp) plus the weight of the HA1 gene (1016 bp) giving band at 6488 bp.

The constructed PCIneo-HA1 recombinant plasmid was sequenced for confirmation of proper ligation at both forward & reverse junctions (Fig 1).

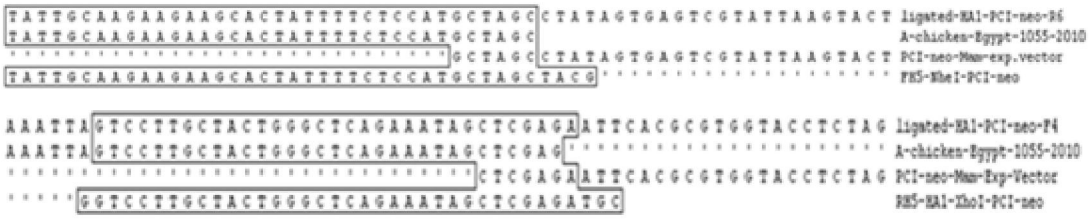

Fig. (1): The upper part showing the forward junction by sequence alignment for the PCI-neo/HA1 construct with HA1 gene & PCI-neo expression vector & FH5-NheI primer and sequencing primer. The lower part showing the reverse junction by sequence alignment for the PCI-neo/HA1 construct with HA1 gene & PCI-neo expression vector & RH5-HA1-XhoI primer and sequencing primer.

The constructed recombinant plasmid DNA (PCI-neo/HA1) was transfected in 293T HEK by electrical method for in vitro expression of HA1 protein to confirm the expression of HA1 protein which detected by SDS-PAGE & detection of mRNA. (Fig 2). The mRNA detection of expressed HA1 protein in transfected 293T HEK by agarose gel electrophoresis showed mRNA of HA1 protein expressed in cell culture at 1016 bp. (Fig 3).

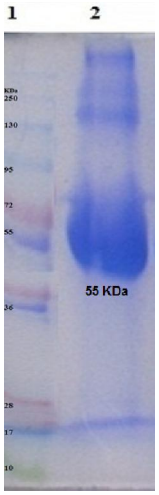

Fig. (2): Coomassie-Stained SDS-PAGE gel showing the HA1 protein was expressed in transfected 293T HEK Lane 1, pageRuler plus prestained protein ladder from 10 KDa to 250 KDa (Fermentas); lane 2: expressed HA1 in T293 human cells at 55 KDa

Evaluation of humoral immune response by haemagglutination inhibition (HI) test

During the first 4 weeks following a single vaccination, the HI titer of the antibody gradually increased every week (0 to 4 $\log_2$ HI titer). There were low detectable prechallenge antibodies to influenza virus antigens in DNA-immunized chickens

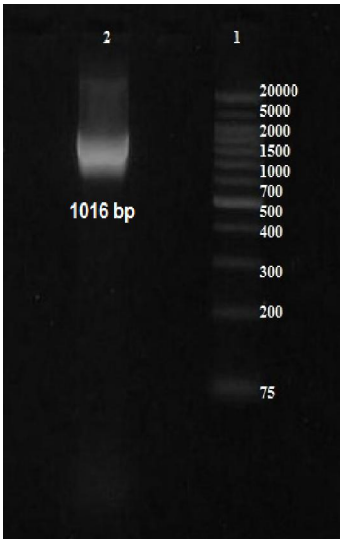

Fig. (3): Agarose gel electrophoresis following RT-PCR of the constructed H5 plasmid DNA vaccine following transfection in 293T (HEK) cell line showed successful expression of H5 mRNA & Bands of the expected size (1016 bp) indicative of HA1 transcripts were detected, Lane 1, GeneRuler™ 1 kb Plus DNA Ladder, fermentas; lane 2: Band of expressed HA1 in T293 human cells.

Evaluation of cell mediated immune response

There was a highly significant increase in the Phagocytic activity (phagocytosis % & phagocytic index) to the HA1 DNA vaccine compared to negative control group ($P<0.05$) ($P=0.000$). The phagocytic activity increased post vaccination up to a peak value reached 2 weeks following the single dose of HA1 DNA vaccine mixed with lipofectamine.

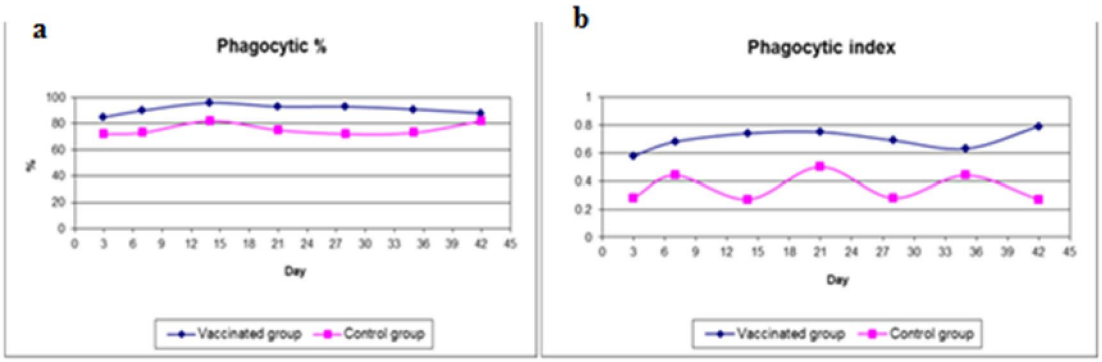

Fig. (4): (a) showing the phagocytic % & (b) showing the phagocytic index in vaccinated & control groups

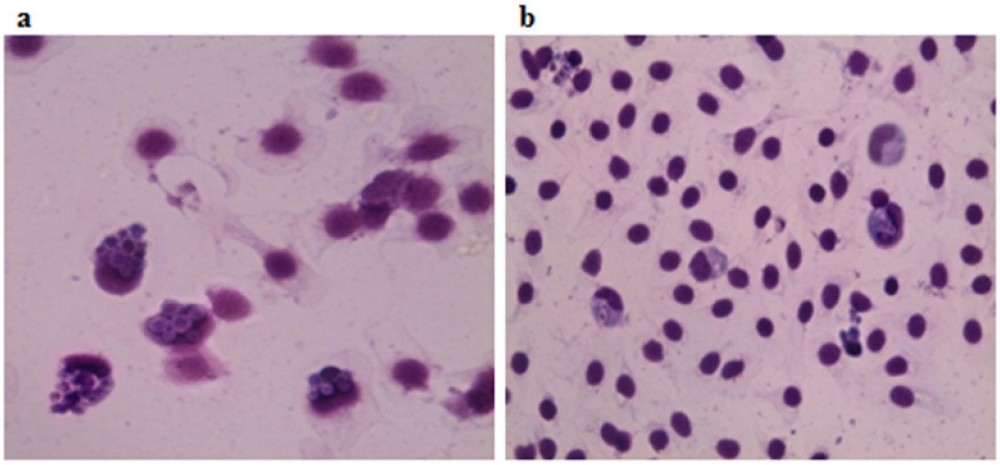

Fig. (5): (a) showing the phagocytic activity in vaccinated group & (b) showing the phagocytic activity in control group

The lymphocyte proliferation increased gradually post vaccination up to a peak value 0.59 compared to negative control which was 0.28 at 7 days following the single dose of H5 DNA vaccine. Fig 6.
There was a highly significant increase in Lymphocyte proliferation in groups vaccinated with H5 plasmid DNA vaccine compared to the negative control group (P=0.000) (P<0.05).

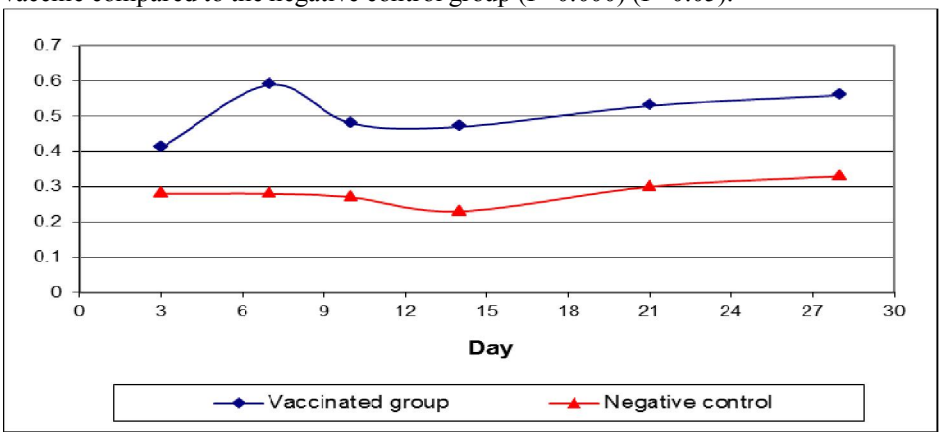

Fig. (6): showing the lymphocyte proliferation assay in vaccinated & control groups

Interleukin 6 (IL6) analysis, the main Th2 cytokine, by two step SYBER Green quantitative real time PCR (qPCR)

The expression level of mRNA encoding IL6 was quantified in chicken lymphocytes following H5 plasmid DNA vaccine inoculation compared with negative & positive controls, Amplification curves of IL6 using SYBER

Green qPCR showed CT of 14.8 for the H5 plasmid DNA vaccinated group, while it was 18.8 for the positive control group.

Protective efficacy of H5 DNA vaccine

Table (1): Protective efficacy of H5 DNA vaccine

Groups	Clinical Signs	Virus shedding[1]	Dead/Total	Survival/total (%)
H5 DNA vaccine	0/10	0/10	4/10	6/10 (60)
Volvac H5N2	6/10	6/10	6/10	4/10 (40)
Positive control	10/10	10/10	10/10	0/10 (0)
Negative control	0/10	0/10	0/10	10/10 (100)

[1]DNA-vaccinated chickens did not show virus shedding after challenge using real time H5 RT-PCR.

4. Discussion

H5N1 avian influenza viruses (AIV) are a disastrous pathogen for domestic poultry that can spread rapidly within and between poultry flocks and is a substantial threat to public health. Efficient vaccination against influenza A virus is a difficult task that has not yet been accomplished by immunologists. Because DNA vaccine seemed to be a promising novel approach for vaccination against influenza A virus **(Montgomery et al., 1993 and Donelly et al., 1995)**, and the HA is no doubt the major target for protective immunity against AIV (**Lee, 2006)**. Since most antigenic and neutralization sites are in the HA1 domain of HA **(Shih et al., 2007),** make using HA1 domain of influenza virus as antigen is of great importance in vaccine development **(Fang-Feng et al., 2009)**. So our ultimate goal was to develop an HA1 based DNA-vaccine to protect chickens against infection and disease with HP H5N1 AIVs. This approach is more cost effective and does not depend upon the use of high pathogenic AIV strains and no need for a BSL3 facility

Neutralization of influenza virus to prevent infection requires antibodies to the HA molecule **(Fynan, et al., 1993, Robinson et al., 1993, and Webster et al., 1994)**. In this study, during the first 4 weeks following a single vaccination, the HI titer of the antibody gradually increased every week (0 to 4 $\log_2$ HI titer). There were low detectable prechallenge antibodies to influenza virus antigens in DNA-immunized chickens & this observation was agreed with previous work **(Kodihalli et al., 1997, Robinson et al., 1993 and Fynan et al., 1993)**, who noted that at 10 days postchallenge, very high antibody titers (GMT, 600 to 800) that were associated with complete protection from lethal virus challenge. This observation indicates a large protective contribution from the B-cell memory response, in keeping with the recognized role of B cells in mediating the immune defenses against influenza virus infection **(Gerhard, 1978)**.

DNA immunizations rely on low numbers of transfected, antigen expressing cells to raise immune responses **(Robinson et al., 1993)**. In our trials, these low numbers of antigen-expressing cells did not induce high-titer antibody responses however they did prime both T-helper and B-cell memory. & this was agreed with **(Fynan et al., 1993b)** who showed that the memory cells appeared to provide protection by supporting the mounting of secondary responses in challenged animals. Evidence for the priming of memory is provided by the DNA inoculations raising antibodies belonging to the IgG isotype. IgG is produced by differentiated plasma cells that have undergone immunoglobulin rearrangements in response to T-cell help **(Abbas et al., 1991)**. Evidence for the mobilization of memory in response to the challenge is found in the rapid increases in serum IgG after challenge **(Fynan et al., 1993b)**.

The potent induction of cell-mediated immunity may reflect endogenous expression of the antigenic protein either in muscle cells or professional APC after I/M immunization **(Donnelly, 1997).** Our results revealed that there was a highly significant increase in the Phagocytic activity (phagocytosis % & phagocytic index) to the HA1 DNA vaccine compared to control group ($P<0.05$) ($P=0.000$). The phagocytic activity increased post vaccination up to a peak value reached 2 weeks following the single dose of HA1 DNA vaccine mixed with lipofectamine (Fig. 4).

Macrophages play an important role in innate and adaptive immunity as professional phagocytes by internalizing and degrading pathogens **[Allen and Aderem, 1996 and Aderem and Underhill, 1999).** Macrophages are known to function as APC, providing cytokines for the activation of T cells. Macrophages also express the co-stimulatory molecules CD80 and CD86, which play a dominant role in T cell activation. Furthermore, these cells may play an important role in T lymphocyte activation through Ag presentation and coligation of the TCR complex **(Michael et al., 1998).**

Our results revealed that there was a highly significant increase in Lymphocyte proliferation to H5 DNA vaccine compared to the control group ($P<0.05$) ($P=0.000$). The lymphocyte proliferation increased post vaccination up to a peak value reached 2 weeks following the single dose of HA1 DNA vaccine (Fig. 6)

Cellular proliferation is an essential feature of the adaptive immune response, T lymphocytes help both humoral and cellular responses, when a cognate antigen is encountered, lymphocytes become activated; undergo clonal proliferation **(Michael et al., 1998).**

The activation of Th cells of the Th2 subset were distinguished by the expression levels of mRNA encoding chicken Th2 cytokine interleukin 6 were quantified in blood lymphocyte following DNA vaccine inoculation Compared with negative controls. This was a significant increase of The Th2 cytokine IL-6 production.

HA1 plasmid DNA vaccine conferred protection (60%) higher than volvac H5N2 inactivated vaccine (40%) (Table 1).

Furthermore we measured virus shedding in the challenge test, cloacal swabs were obtained from all chickens on 2nd day after challenge, the virus titer represented the protection. There was no virus shedding in DNA-vaccinated chickens after challenge using real time H5 RT-PCR.

The simple and direct criterion for evaluating whether the H5 DNA vaccine offered protection was the absence of disease signs, virus shedding and deaths in vaccinated chickens after lethal challenge **(Jiang et al., 2006)**.

In our study although immunization with HA DNA induced weak antibody responses, it generated potent cell-mediated immunity and since it has been suggested that both arms of the immune response can contribute to protection against infection, it was important to establish if the HA DNA vaccine could confer protection against influenza virus infection. It is worth noting that protection against a lethal challenge in the absence of HI titers can be facilitated by cell-mediated immunity, which is consistent with previous reports **(Park et al., 2009)**.

Alternatively, the protection observed with the HA DNA vaccine may be mediated solely through the induction of cell-mediated immunity. This conclusion is consistent with reports which have demonstrated that mice that lack mature B cells and do not secrete immunoglobulin can clear an influenza virus infection from the respiratory tract **(Topham et al., 1996)** & also agreed with **(Robinson et al., 1997)** who showed that the mechanisms used by an organism against influenza virus infection usually involve cellular and humoral responses. In general, the sufficient and differential cytotoxic T lymphocyte (CTL) immune responses restrict the replication of infected virus and probably eliminate viral infection quickly.

Despite the strong potent induction of cell-mediated immunity influenza virus-specific T cell response in the chickens vaccinated with DNA vaccine 2 weeks following a single dose of HA DNA vaccine, higher dose concentration of HA1-DNA vaccine were required for providing complete protection of chickens from lethal dose of H5N1 AIV.

This was agreed with **(Gurunathan et al., 2000)** who reported that immunization via i/m route require 10-100 µg of plasmid DNA to induce immune response & provide protection. Also these results agreed with that intramuscular injection require larger amounts of DNA for a measurable response **(Fynan et al., 1993 a, b)**.

In conclusion, results presented in this study showed that HA1 based DNA vaccine is a promising novel approach for vaccination against avian influenza virus as it induced potent cell mediated immunity which is sufficient for protection against lethal challenge despite of low antibody titers, also HA1 based DNA vaccine induced the Th2 related cytokine (IL6) which promotes B cell proliferation & differentiation & mediate the humoral immune response.

Corresponding Author:
Mady W. H.
National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, P.O. Box 264-Dokki, Giza-12618, Egypt, Tel: 202 33380121, Fax: 202 33370957, Mobile: 01096105189 – 01147276255
Email: wesammady83@hotmail.com, wesammady@gmail.com

Conflict of interests:

The authors reveal that there are no any possible conflicts of interest in their submitted manuscripts. All authors do not have any direct or indirect financial relation with the trademarks mentioned in this paper that might lead to a conflict of interest.

References

1. Abbas, A. K., Lichtman, A. H. & Pober, J. S., (1991). Cellular and Molecular Immunology (Saunders, Philadelphia), pp. 187- 197.
2. Abdelwhab EM, Hafez HM. An overview of the epidemic of highly pathogenic H5N1 avian influenza virus in Egypt: epidemiology and control challenges. Epidemiol Infect 2011

3. Aderem, A, Underhill, DM. (1999). Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. 17:593-623.
4. Allen, LA, Aderem, A. (1996). Mechanisms of phagocytosis. Curr. Opin. Immunol. 8:36-40.
5. Aly MM, Arafa A, Hassan MK. Epidemiological findings of outbreaks of disease caused by highly pathogenic H5N1 avian influenza virus in poultry in Egypt during 2006. Avian Dis 2010;52:269–77.
6. Arafa A, Suarez DL, Hassan MK, Aly MM. Phylogenetic analysis of HA and NA genes of HPAI-H5N1 Egyptian strains isolated from 2006 to 2008 indicates heterogeneity with multiple distinct sublineages. Avian Dis 2010;54:345–9.
7. Balish AL, Davis T, Saad MD, El-Sayed N, Esmat H, Tjaden JA, et al. Antigenic and genetic diversity of highly pathogenic avian influenza A (H5N1) viruses isolated in Egypt. Avian Dis 2010; 54:329–34.
8. Barry Ghamon and John, R. Gilsson (1988): In vitro microbicidal activity of avian peritoneal macrophages. Avian Diseases, 33: 177-181.
9. Donnelly, J. J. (1997). DNA vaccines. Annual Reviews in Immunology 15, 617-648.
10. Donelly J. J., Friedman A, Martinez D, Montgomery DL, Shiver JW, Motzel SL, Ulmer JB. (1995). Preclinical efficacy of a prototype DNA vaccine: enhanced protection against antigenic drift in influenza virus. Nat Med; 1:583-587.
11. Fang-Feng Chiu, Nandini Venkatesan, Chia-Rong Wu, Ai-Hsiang Chou, Hsin-Wei Chen, Shu-Pei Lian, Shih-Jen Liu, Chin-Cheng Huang, Wei-Cheng Lian, Pele Chong, Chih-Hsiang Leng, (2009). Immunological study of HA1 domain of hemagglutinin of influenza H5N1 virus Biochemical and Biophysical Research Communications 383 (2009) 27–31
12. Fynan, E. F., H. L. Robinson, and R. G. Webster. (1993a). Use of DNA encoding influenza hemagglutinin as an avian influenza vaccine. DNA Cell Biol. 12:785–789.
13. Fynan, E. E, R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. (1993b). DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. Proc. Natl. Acad. Sci. U.S.A. 90:11478-11482.
14. Gerhard, W. (1978). The analysis of the monoclonal immune response to influenza virus. III. The relationship between stimulation of virus primed precursor B-cells by heterologous virus and reactivity of selected antibodies. J. Immunol. 120:1164–1168.
15. Gurunathan, S., Klinman, D.M. and Seder, R.A. (2000). DNA vaccines: immunology, application, & optimization. Annual review of immunology 18, 927-974
16. Jalilian, B., Omar, A. R., Bejo, M. H., Alitheen, N. B., Rasoli, M., & Matsumoto, S. (2010). Development of avian influenza virus H5 DNA vaccine and MDP-1 gene of Mycobacterium bovis as genetic adjuvant. Genetic Vaccines and Therapy, 8, 4. BioMed Central.
17. Jiang Yong-Ping1, Zhang Hong-Bo1, Li Cheng-Jun1, Bu Zhi-Gao2, Deng Guo-Hua1, Yu Kangzhen3 and Chen Hua-Lan1, (2006). Comparison of protective effects among H5 subtype avian influenza DNA vaccines with optimized HA gene and expressive vectors Journal of Agricultural Biotechnology, 14(3): 301–306
18. Kilany, W. H., Abdelwhab, E. M., Arafa, A.-S., Selim, A., Safwat, M., Nawar, A. A., Erfan, A. M., et al. (2011). Protective efficacy of H5 inactivated vaccines in meat turkey poults after challenge with Egyptian variant highly pathogenic avian influenza H5N1 virus. Veterinary Microbiology, 150(1-2), 28-34.
19. Kodihalli S., Joel R. Haynes, Harriet L. Robinson, and Robert G. Webster (1997). Cross-Protection among Lethal H5N2 Influenza Viruses Induced by DNA Vaccine to the Hemagglutinin, journal of virology, May 1997, p. 3391–3396
20. Laemmli U. K. (1970): Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227: 680-685.
21. Lee CW, Senne DA, Suarez DL (2006). Development and application of reference antisera against 15 hemagglutinin subtypes of influenza virus by DNA vaccination of chickens. Clin Vaccine Immunol 13:395–402.
22. Lewis DB. (2006). Avian flu to human influenza. Annual Review of Medicine, 57:139-154.
23. Madbouly H. M., Orkhan M. H. and Hussein A. S. (2012): Comparative molecular Analysis of Egyptian strains of Highly Pathogenic Avian influenza (HPAI) virus H5N1 isolated during 2006 and 2007. Journal of American Science 2012: 8 (10)
24. Michael A. Chattergoon, Tara M. Robinson, Jean D. Boyer and David B. Weiner (1998). Specific Immune Induction Following DNA-Based Immunization Through In Vivo Transfection and Activation of Macrophages/Antigen-Presenting Cells http://www.jimmunol.org/content/160/12/5707 J Immunol; 160:5707-5718.
25. Montgomery DL, Shiver JW, Leander KR, Perry HC, Friedman A, Martinez D, Ulmerl JB. (1993).

Heterologous and homologous protection against influenza A by DNA vaccination: optimization of DNA vectors. DNA Cell Biol; 12:777-783.
26. OIE Terrestrial Manual (2009): Avian influenza. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2. 3. 4.
27. Park KS, Lee J, Ahn SS, Byun YH, Seong BL, Baek YH, et al. (2009). Mucosal immunity induced by adenovirus-based H5N1 HPAI vaccine confers protection against a lethal H5N2 avian influenza virus challenge. Virology; 395(2):182–9.
28. Rafael Fernández-Botrán, Ph.D., Zeshan Ahmed, Ph.D., Fabián A. Crespo, Ph.D., Chandler Gatenbee, M.S., John Gonzalez, M.S., Dennis W. Dickson, M.D., and Irene Litvan, M.D., (2011): cytokine expression and microglial activation in progressive supranuclear palsy. 17(9): 683–688.
29. Reed, L.J. and H. Muench. (1938): A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.
30. Richardson, M.D. and Smith, H. (1981): Resistance of virulent and attenuated strains of C. albicans to intracellular killing by human and mouse phagocytes. J. Infect. Dis., 144: 557-565.
31. Robinson, H. L., Hunt, L. A. & Webster, R. G. (1993). Protection against a lethal influenza virus challenge by immunization with a haemagglutinin expressing plasmid DNA. Vaccine 11, 957-960.
32. Robinson HL, Boyle CA, Feltquate DM, Morin MJ, Santoro JC and Webster RG. (1997). DNA immunization for influenza virus: studies using hemagglutinin- and nucleoproteinexpressing DNAs. Journal of Infectious Disease 176(Suppl. 1): S50–55.
33. Shih, A. C.-C., Hsiao, T.-C., Ho, M.-S., & Li, W.-H. (2007). Simultaneous amino acid substitutions at antigenic sites drive influenza A hemagglutinin evolution. Proceedings of the National Academy of Sciences of the United States of America, 104(15), 6283-6288.
34. Spackman, E.; Senne, D. A.; Myers, T. J.; Bulaga, L. L.; Garber, L. P.; Perdue, M. L.; Lohman, K.; Daum, L. T. and Suarez, D. L. (2002): Development of a Real-Time Reverse Transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J. Clin. Microbial., 40(9) : 3256-3260.
35. Topham, D. J., Tripp, R. A., Hamilton-Easton, A. M., Sara, S. R. & Doherty, P. C. (1996). Quantitative analysis of the influenza virus specific CD4- T cell memory in the absence of B cells and Ig. Journal of Immunology 157, 2947-2952.
36. Voeten JTM, Brands R, Palache AM, Van Scharrenburg GJM, Rimmelzwaan GF, Osterhaus ADME. (1999) Characterization of high-growth reassortant influenza A viruses generated in MDCK cells cultured in serum-free medium. Vaccine, 17(15-16):1942-1950.
37. Webster, R. G., E. F. Fynan, J. C. Santro, and H. L. Robinson. (1994). Protection of ferrets against influenza challenge with a DNA vaccine to haemagglutinin. Vaccine 12:1495–1498.

5/2/2013