Research Article

Construction and Protective Efficacy of Egy-H5 DNA Vaccine from Local Egyptian strain H5N1 using Codon Optimized HA gene

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Abstract | The incursion of highly pathogenic avian influenza virus (HPAIV) strain H5N1 in 2006 in Egypt caused severe economic losses for both commercial and backyard poultry production sectors. Owing to public health risk, effective control of the virus in imperative. DNA vaccination is a promising new approach for the prevention and treatment of many viral diseases because of its ability to induce both humoral and cellular immune responses against antigens encoded by recombinant DNA. The goal of this study was to construct Egy-H5 plasmid DNA-based vaccine against a currently circulating avian influenza virus H5N1 strain in Egypt targeting the HA gene. The HA gene of AIV Egyptian strain H5N1 was codon optimized for chicken biased codon and sub-cloned into pCAGGS mammalian expression vector under the control of chicken β-actin promoter. The construct was transfected into 293T HEK (Human Embryonic Kidney) cell line for in vitro expression of the recombinant plasmid DNA. The confirmation of H5 protein expression was performed using SDS-PAGE followed by Western blotting and immunofluorescence assays. The humoral immune response was evaluated by intramuscular immunization of specific pathogen free (SPF) chickens with two different concentrations of Egy-H5 plasmid DNA 15µg and 60 µg. Results demonstrate that chickens developed detectable HI antibody titers up to $6\log_2$ within two weeks post-booster vaccination. The challenge experiment was performed using isolate A/chicken/Egypt/A6/2011 (H5N1) with concentration of 10^5 EID_{50} to evaluate the protective efficacy of the Egy-H5 plasmid DNA vaccine. The Egy-H5 plasmid DNA vaccine induced complete protection (100%) and there was no virus shedding in all chickens immunized with the Egy-H5 plasmid DNA vaccine. Taken together, the presented immunization approach is an alternative strategy to not only control the infections in chicken but also to safeguard public health.

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Introduction

H5N1 HPAI outbreak was first recorded in Egypt during mid-February 2006 and has reportedly caused severe economic losses in commercial and backyard poultry production sectors (Aly et al., 2006 a; b) as well as it possesses considerable zoonotic potential. Since its identification, the virus

remained endemic and threat to both public health and the poultry industry (Aly et al., 2008). Previous studies on Egyptian H5N1 viruses indicated high evolution rate with the emergence of vaccine escape mutants, and genetic and antigenic variations among viruses (Arafa et al., 2016). Some of these mutations can affect human transmission and reflects high incidence of human cases with H5N1 in Egypt during last few years (Balish et al., 2010; Arafa et al., 2010; Cattoli et al., 2011; Arafa et al., 2012).

Several experiments have been performed to monitor the vaccine efficacy of different inactivated commercial and experimental H5-vaccines to protect against challenge viruses derived from different circulating Egyptian strains. Most of these studies indicate insufficient protection of some of these vaccines and directed toward the continuously drifting Egyptian viruses (Kilany et al., 2011; Grund et al., 2011; Connie et al., 2013). Therefore, a vaccine that could protect chickens from a lethal infection and prevent the spread of the virus is urgently needed. Development of cost-effective avian influenza (AI) vaccine is a priority to prevent pandemic flu outbreaks. DNA-based immunization offers a promising novel approach for vaccination against avian influenza viruses, as it is capable to induce both humoral and cellular immunological responses (Stachyra et al., 2016). The haemagglutinin (HA) surface glycoprotein is a major contributor to induction and eliciting protective neutralizing antibodies against avian influenza viruses administered via vaccination or natural infection (Madhu et al., 2014; Baibaswata et al., 2010). In this study, HA protein was targeted for the development and assessment of protective efficacy of a DNA-vaccine in chicken. Findings of this study highlight the applied approach an alternative strategy to contain viral infections especially in endemic situations.

Material and Methods

Virus strains

Local Egyptian strain (A/duck/Egypt/13149S/2013 (H5N1) of highly pathogenic avian influenza virus was provided by Reference Laboratory for Veterinary Quality Control on Poultry Production (RLQP). The virus characteristics can be acquired from PubMed using KJ522744 accession number.

Construction of Egy-H5 plasmid DNA vaccine

The full coding HA sequence of H5N1 virus strain was codon optimized for chicken biased codon using the DNASTAR from a codon usage database (Opti-HA) and the Kozak sequence (GCCGCCACC) was added before the ATG start codon. The synthetic opti-HA gene that was chemically synthesized by Genscript (Nanjing Co., Ltd., China) and was subcloned into the pCAGGS mammalian expression vector under the control of chicken β actin promoter provided by Harbin Veterinary Research Institute, China.

The specific H5 primers were designed at Harbin veterinary research institute, China, containing the sequence of the restriction endonuclease site of EcoRI inserted upstream to the Kozak sequence in the upstream primer and the sequence of the restriction endonuclease site of the XhoI inserted to the 5' end of the downstream primer (the primers available upon request). The recombinant plasmid DNA (pCA-Egy-H5) was then transformed into Trans5 α *E. coli* competent cells and the screening of positive colonies was performed by PCR using pre-designed H5 primers. pCA-Egy-H5 plasmid DNA was then extracted using Axygen mini prep plasmid extraction kit and confirmed by digestion analysis using EcoRI and XhoI restriction endonucleases and sequencing.

In vitro expression of pCA-Egy-H5 plasmid DNA vaccine

Transfection of pCA-Egy-H5 plasmid into 293T HEK cells was performed using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. A total of 4μg plasmid DNA was mixed with Lipofectamine in a ratio of 2.5 μl Lipofectamine-2000/μg plasmid DNA and incubated for 30 minutes at room temperature before adding to cells. After 6-12 hours, the transfection mixture was aspirated and replaced by optiMEM and incubated for 48 hours. Immunofluorescence assay (IFA) and Western blotting were performed for detection and confirmation of H5 HA protein expression.

Protein analysis and in-vitro confirmation of protein expression

Immunofluorescence assay (IFA): The optimem was aspirated 48 hours post-transfection and cells were fixed by 4% paraformaldehyde for 30 minutes at room temperature. Cells were washed with PBS before adding the primary antibody (diluted 1:200 in blocking solution BSA, Bovine serum albumin) and the cells were incubated for 1 hour at 37°C followed by wash-

ing with PBS. The secondary antibodies were diluted in 1:500 in BSA for 1 hour at 37°C followed by washing with PBS. The DAPI (100ng/ml) was then added for 10 minutes at room temperature and the cells were washed with PBS before examining under the fluorescent microscope.

Western blotting: Cells were transfected for 48 hours before lysing and separation on 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred and blotted to nitrocellulose membrane and blocking was performed in 5% skimmed milk at 4°C for overnight. The membrane was then hybridized with chicken H5-AIV specific antiserum as primary antibody (diluted 1:200) and detection by anti-secondary antibodies (diluted 1:1000).

Evaluation of pCA-Egy-H5 plasmid DNA vaccine efficacy

SPF chickens of 3 weeks old were immunized with two different concentrations of pCA-Egy-H5 plasmid (15 µg and 60 µg); each concentration was injected in a group of 10 chickens by intramuscular injection of 0.2 ml of plasmid DNA into the thigh muscle. Three weeks later, chickens were immunized with a booster dose of the same concentration of the first dose.

The protective efficacy of the pCA-Egy-H5 plasmid DNA vaccine was investigated by challenge test using A/chicken/Egypt/A6/2011(H5N1) ($10^5 EID_{50}$) intranasally two weeks after the booster immunization. The chickens were observed daily for 2 weeks for deaths and clinical signs, oropharyngeal and cloacal swabs were collected at 3, 5 and 7 days post-challenge (p.c.) for virus titration in eggs for evaluation of virus shedding. Sera were collected weekly post immunization for evaluation of the humoral immune response by using the hemagglutination inhibition (HI) test according to OIE recommendation (OIE, 2009).

Ethical statement

All institutional and national guidelines for the care and use of laboratory animals were followed. This article does not contain any studies with human subjects performed by any of the authors.

Results and Discussion

Construction of Egy-H5 Plasmid DNA vaccine

Screening of positive clones containing the Egy-H5 plasmid construct was performed by PCR using H5-specific primers designed at Harbin Veterinary Research Institute, China. The electrophoresis showed the specific band at the expected size of 1700 bp for the inserted opti-HA gene (Figure 1a). The pCAGGS-opti HA plasmid was extracted and confirmed by double digestion using EcoRI and XhoI restriction endonucleases (Figure 1b).

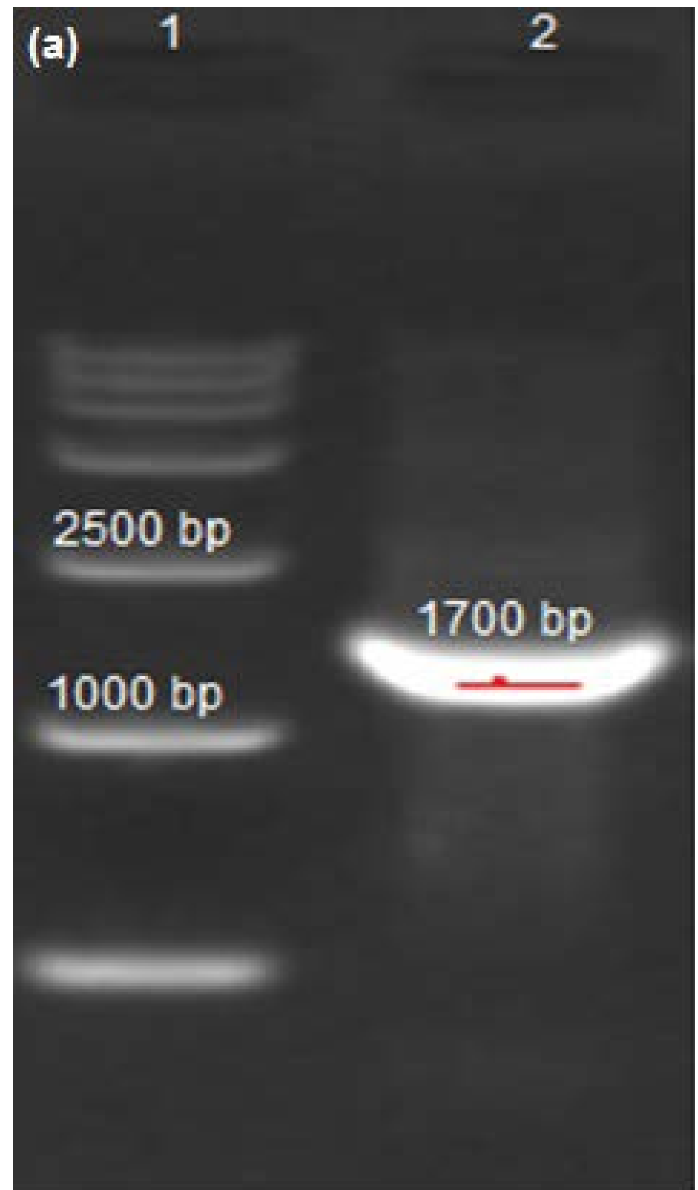

Figure 1a: *Agarose Gel electrophoresis showing the screening of positive clones, Lane 1 is the molecular weight marker and lane 2 is the inserted opti-HA at the expected weight 1700 bp.*

Protein analysis and in-vitro confirmation of protein expression

The HA protein expression in 293T HEK cells was detected and confirmed by indirect immunofluorescence assay (IFA) and Western blotting (WB). As shown in Figure 2a and 2b, the transfected 293T cells with the pCA-Egy-H5 revealed obverable im-

mune-staining of the H5 antigen, whereas no staining was observed in non-transfected cells. The H5 HA protein expression in 293T HEK cells was separated by SDS-PAGE and confirmed by Western blotting using H5-specific antibodies and the expressed H5 HA protein showed specific band (Figure 3).

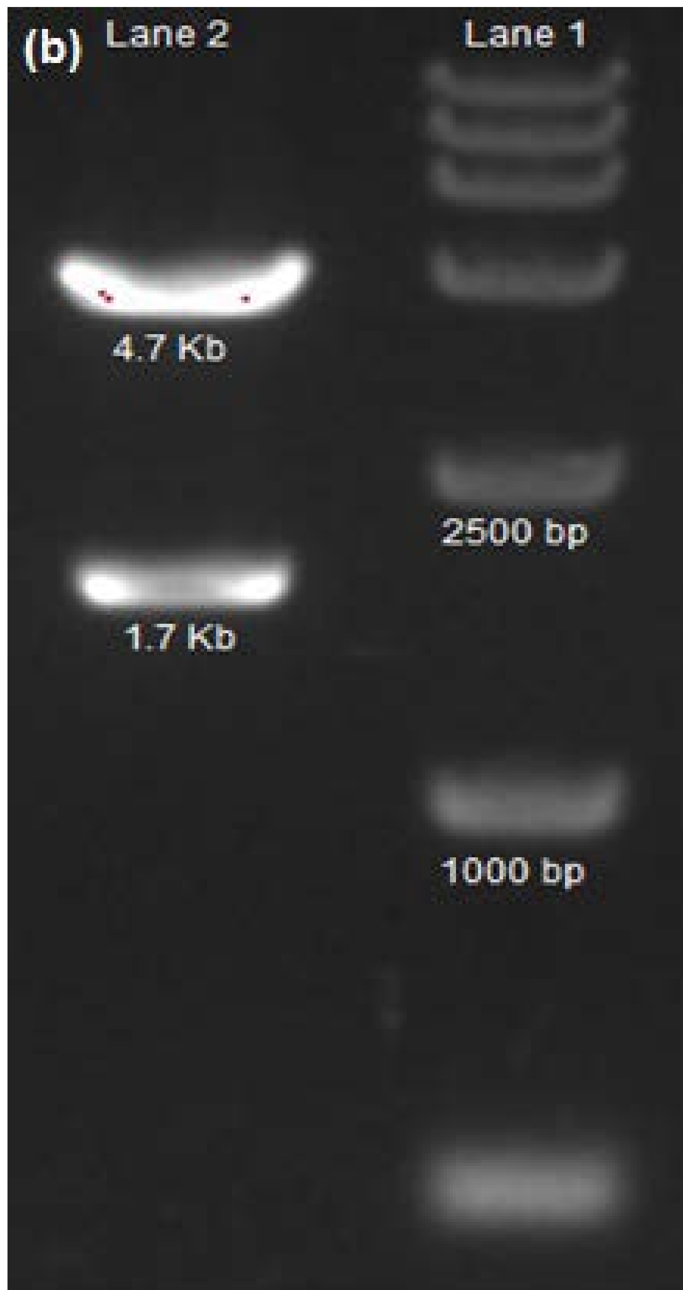

Figure 1b: *Agarose gel electrophoresis showing the pattern of the digestion analysis. Lane 1 is the molecular weight marker and Lane 2 is the double digested pCA-Egy-H5 plasmid DNA showing 2 bands, the upper band is the pCAGGS plasmid at 4.7 Kb and the Lower band is the opti HA at 1.7 Kb.*

Antibody response induced by Egy-H5 plasmid DNA vaccine

Evaluation of the humoral immune response was performed by detecting HI antibodies in sera collected from chicken. Employing HI assay, the chickens immunized with the Egy-H5 plasmid DNA showed detectable antibody titers. Two weeks post-booster application, the mean HI antibody titers were 2.7 log2 and 3.7 log2 in the 15 µg and 60 µg plasmid-inoculated groups, respectively. After the challenge, the mean HI antibody titers were reached at 5.8 log2 and 5.6 log2 in the 15 µg and 60 µg plasmid-inoculated groups, respectively (Figure 4).

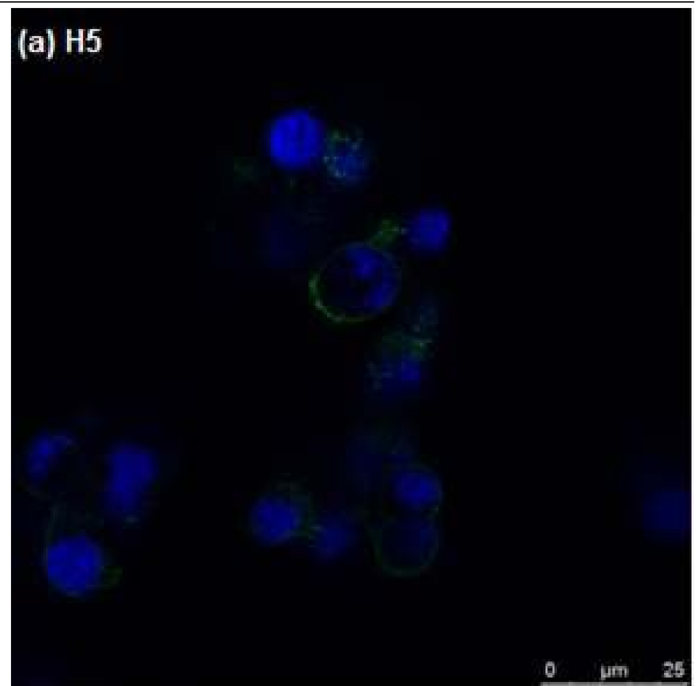

Figure 2a: *The 293T HEK cells transfected with pCA-Egy-H5 showing the bright specific fluorescence.*

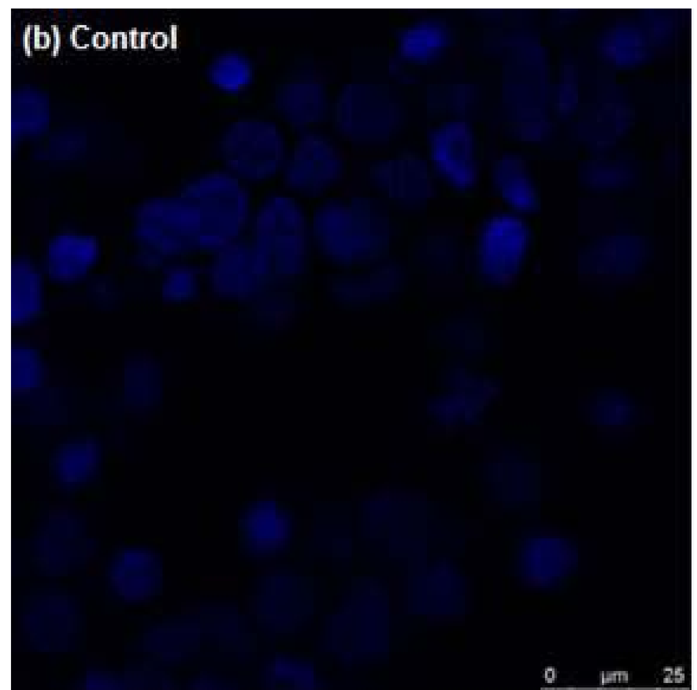

Figure 2b: *The negative Control 293T HEK cells not transfected did not show any fluorescence.*

Protective efficacy of Egy-H5 plasmid DNA vaccine

Table 1: The virus titrations from swabs post H5N1 virus challenge[A].

Vaccines	Doses	Virus isolation from swabs: shedding/total (titer, $\log_{10}EID_{50}$/ml)						Survival/ total
		Day 3		Day 5		Day7		
		Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	
pCA-Egy-H5	15μg	0/10	0/10	0/10	0/10	0/10	0/10	10/10
	60μg	0/10	0/10	0/10	0/10	0/10	0/10	10/10
Control	PBS	1/1(1.25)[B]	0/1	/	/	/	/	0/10

[A]: *Groups of 3-week-old SPF chickens were inoculated intramuscularly with 15 μg and 60 μg of pCA-Egy-H5 in 100 mL of PBS or with 100 mL of PBS as a control. Animals were challenged intranasally with 10^5EID_{50} of A/chicken/Egypt/A6/2011(H5N1) virus. Swabs were suspended in 1 mL of PBS and titrated for virus shedding in eggs at an initial dilution of 1:10, or undiluted if negative at the lowest dilution. / All birds died;* [B] *:Swabs from chickens that died before day 3 were not collected.*

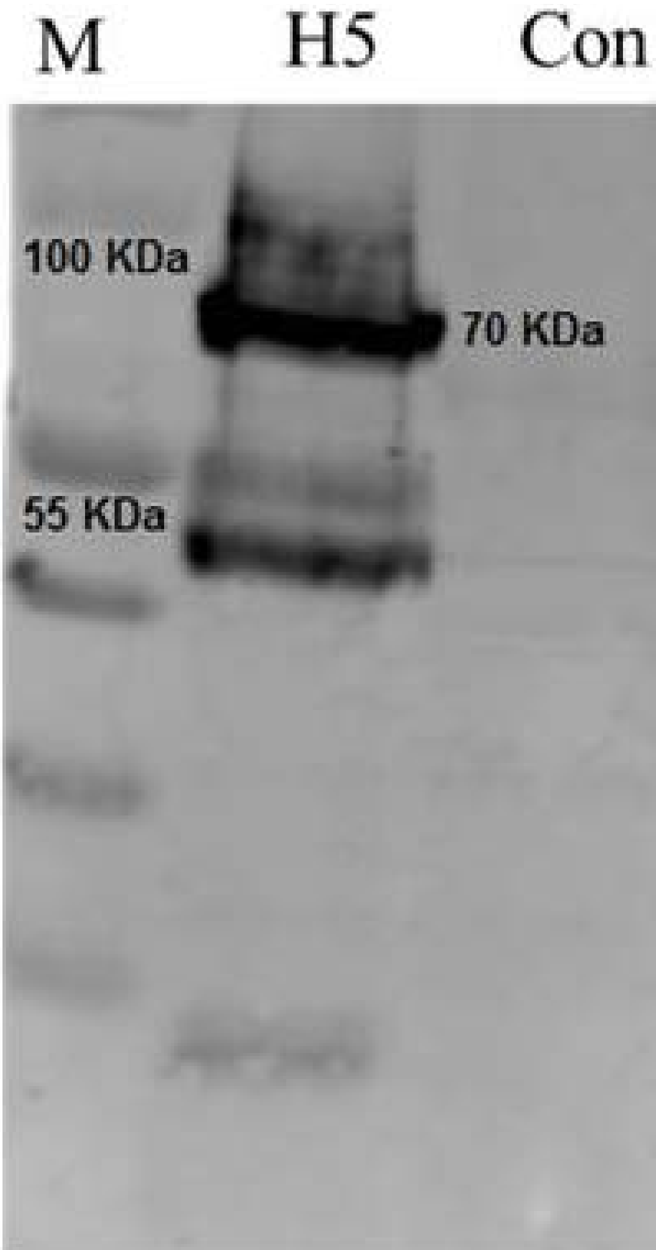

Figure 3: *Showing the western blotting of the expressed H5 protein in 293T HEK cells, M is the molecular weight marker, H5 is the expressed HA protein showing the specific band at 70 KDa, and Con = the cell negative control not transfected.*

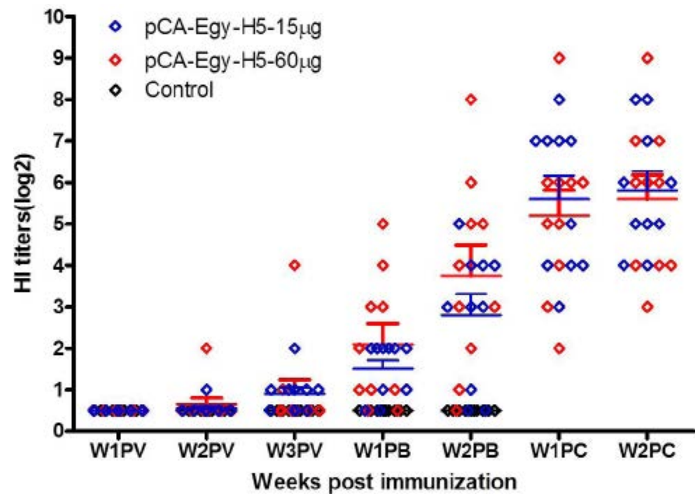

Figure 4: *The HI antibody titers induced by pCA-Egy-H5 post immunization. W1PV: 1-week post vaccination; W2PV: 2 weeks post vaccination; W3PV: 3 weeks post vaccination; W1PB: 1-week post boost; W2PB: 2 weeks post boost; W1PC: 1-week post challenge; W2PC: 2 weeks post challenge.*

in chickens against the H5N1 HPAI virus challenge
The protective efficacy of the constructed pCA-Egy-H5 plasmid DNA vaccine after challenging the immunized SPF chickens intranasally with H5N1 HPAI virus strain A/chicken/Egypt/A6/2011(H5N1) (10^5EID_{50}) revealed that all of the control chickens died within three days post-challenge. However, all of the vaccinated chickens were survived from a lethal challenge and no clinical signs and deaths were observed. The virus shedding was evaluated by virus isolation and titration from oropharyngeal and cloacal swabs in SPF ECE. Results showed the absence of virus shedding, and all chickens immunized with Egy-H5 DNA vaccine was virus free (Table 1).

H5N1 HPAI is a disastrous pathogen for domestic poultry that can spread rapidly within and between poultry flocks causing a substantial threat to public health. Since the incursion of highly pathogenic avian influenza virus (HPAIV) of subtype H5N1 of phylogenetic clade 2.2, subclade 2.2.1, into Egypt in 2006, it caused severe economic losses in the commercial and backyard sectors of poultry production

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in Egypt (Aly et al., 2006a; b). The avian influenza vaccines used in Egypt which are inactivated whole virus vaccines resulted in poor protection against the existing AI virus strains in Egyptian chicken farms, and as a result, economic losses will be continuous so the development of AI vaccine from the same strain circulating in the field is of great need to control the disease. However, some recent H5 vaccines were developed from local strains but frequent mutations in the Egyptian viruses needs more rapid response strategy for vaccine seed selection and production that remains very hard and time consuming by using the conventional inactivated vaccines.

DNA vaccine is a promising and cost-effective strategy for vaccination against influenza A viruses that can be easily produced on a large scale. The procedure of cloning, expression, and manufacture of rHA influenza vaccine allowing the rapid reconstruction of new vaccinal seed strain based on the available epidemiological data (Stachyra et al., 2016). Veterinary officials would be better able to respond in the event of the emergence of a new epizootics or frequent outbreaks arising from new emerging strains of H5N1 HPAI viruses.

DNA vaccines designed to prevent infection most commonly aim to induce the neutralizing antibody. To produce neutralizing antibody, the most appropriate target is usually a protein on the surface of the pathogen in its extracellular state (Saltzman et al., 2006). In avian influenza virus, HA and NA proteins are primary targets of the protective antibody responses; antibodies against HA neutralize virus infectivity (Subbarao et al., 2006). The HA is the major target for protective immunity against avian influenza viruses and the antibodies against this surface glycoprotein can provide protection by blocking virus attachment and entry (Lee et al., 2006). Because HA protein is a major viral surface antigen against which the neutralizing antibodies are elicited, thus the recombinant HA is an ideal target as a candidate avian influenza vaccine.

In this study, we developed the pCA-Egy-H5 plasmid DNA vaccine from local Egyptian strain of H5N1 HPAI circulating in the field. For the construction of the plasmid DNA, the H5 gene was codon optimized to the chicken biased codons for chicken usage with the Kozak sequence (GCCGCCACC) inserted before the ATG start codon. The synthetic opti-HA was then inserted into the pCAGGS plasmid under the control of chicken β actin promoter. pCA-Egy-H5 plasmid DNA vaccine was able to elicit detectable and measurable HI antibody titers against H5N1 AI virus up-to 5 log2 after booster immunization and completely protected the chickens from lethal H5N1 HPAI virus.

It has been proven earlier that codon optimization of HA gene together with the effect of the pCAGGS plasmid vector give better immune response than the other vectors and this is due to the activity of the promoter which is the chicken β actin promoter which leads to better gene expression and influences the immune response (Jiang et al., 2007). The antibody response in chickens induced by the plasmids containing the optiHA gene were better than those induced by the plasmids containing the wild-type HA gene and the codon optimization of the HA gene to the chicken biased codons for chicken usage allow for the increase of the level of HA gene expression in the chickens (Jiang, 2007).

According to Shan et al. 2011, it has been demonstrated that the pCAGGS plasmid vector encoding codon optimized HA together with insertion of a Kozak sequence, was able to elicit measurable HI antibody responses in SPF chickens as the Kozak consensus sequence plays a major role in the initiation of the translation process and it is important and required for optimal translational the efficiency of expressed mammalian genes (Kozak 1987; 1997).

The protective efficacy of the Egy-H5 plasmid DNA vaccine was evaluated by performing the challenge where chickens were challenged intranasally with H5N1 HPAI strain and the were observed daily for deaths and clinical signs for two weeks post challenge. All the immunized chickens with Egy-H5 plasmid DNA vaccine were completely protected against the lethal H5N1 HPAI, no deaths and no clinical signs were observed. Furthermore, we evaluated the virus shedding by inoculation of oropharyngeal and cloacal swabs collected at 3rd, 5th, and 7th days post-challenge in SPF ECE for virus isolation and titration, our results revealed that all chickens immunized with the Egy-H5 DNA vaccine was virus free and no virus shedding was observed.

In this study the HI antibody titers were adequate (5 log2) but the immunized chickens were completely

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protected from the lethal H5N1 HPAI virus and this agrees with Jiang (2006) as they reported that the HI antibody could not be or was marginal, detected in some singly immunized chickens. However, these chickens still survived the lethal challenge. This phenomenon might result from the CTL responses induced by the HA DNA vaccine.

Taken together, the reported pCA-Egy-H5 plasmid DNA vaccine could completely protect the chickens from a lethal H5N1 highly pathogenic avian influenza virus with the absence of virus shedding and absence of clinical signs.

Compliance with Ethics Guidelines

The authors declare no conflict of interest.

Authors Contribution

W. H. Mady, A. Arafa and Yongping Jiang conceived the study; W. H. Mady, Bing Liu and Dong Huang performed the research; W. H. Mady, Bing Liu, Dong Huang, A. Arafa, M. K. Hassan, M. M. Aly, Pucheng Chen, Yongping Jiang and Hualan Chen were involved in drafting the work and revising it critically for important intellectual content. W. H. Mady, A. Arafa and Yongping Jiang wrote the manuscript. And W. H. Mady, Bing Liu, Dong Huang, A. Arafa, M. K. Hassan, M. M. Aly, Pucheng Chen, Yongping Jiang, Hualan Chen compiled the final approved version to be published.

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