A DNA vaccine against an H1N1 avian influenza virus induced humoral and cell-mediated immunity in SPF chickens

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The development of cost-effective avian influenza (AI) vaccine is a priority to prevent pandemic flu outbreaks. DNA-based immunization offers a promising strategy to prevent viral diseases. In this study, immunological response induced by an experimental DNA vaccine against AIV in specific pathogen-free chickens was investigated. The vaccine consisted of the entire HA gene of an AIV H1N1 subtype Alabama strain (A/blue-winged teal/ AL/167/2007) cloned into the pcDNA6.2 DNA eukaryotic expression vector. The *in vitro* expression of the DNA vector was confirmed in COS-7 cells by indirect immunofluorescence. The *in vivo* expression of the cloned gene was confirmed in intramuscularly (IM) vaccinated chicken breast muscles by immunohistological analysis. Results indicated that pcDNA6.2 vector was as effective as a commercial inactivated H1N1 AIV vaccine given IM in inducing hemagglutination inhibition (HI) antibody. This vector produced lower neutralization antibody than the commercial vaccine. The Th1 (IFN-γ, IL-2) and Th2 (IL-4, IL-6, IL-10) cytokine profiles in vaccinated birds were analyzed by reverse transcriptase and quantitative real-time PCR (qRT-PCR). The Th1-like immune response of the experimental vaccinated birds was high as shown by levels of interferon gamma (INF-γ) and interleukin-2 (IL-2), but not IL-4, IL-6 or IL10. Our results suggest that the experimental DNA vaccine produced measurable humoral and cellular immune responses in chickens.

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Introduction

Avian influenza (AI) is a viral disease that can spread rapidly within and between poultry flocks. AI viruses (V) are subdivided using the presence of their hemagglutinin (HA) and neuraminidase (NA) surface proteins. According to molecular characterization and pathotyping, AIV can be further divided into high pathogenic (HPAI) and low pathogenic (LPAI). AIV of the H1N1 subtype are classified as LPAI, since they cause no or mild respiratory diseases in chickens. These viruses infect a wide variety of species, including chickens, quail, turkeys, ducks, geese, pigs, and humans [1]. The recent pandemic outbreak of influenza virus in humans caused by the H1N1 contained part of avian-originated HA sequence [2].

There is need to prevent the spread of the avian H1N1 virus to swine or humans to prevent viral genome recombination. This has been done using quarantine measures, culling of infected poultry flocks, and improved biosecurity. However, vaccination is an important tool to prevent infection and disease to limit outbreaks and prevent the spread between species.

Several strategies have been tried to develop effective AIV vaccines: such as recombinant vaccines, subunit hemagglutinin inactivated proteins, reverse genetic vaccines, and DNA

vaccines [3-6]. 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, herpes simplex, rabies, human immunodeficiency virus in different animal models [7-13]. The DNA-based immunization has been shown to induce cell mediated immune (CMI) and humoral immune responses in mice, duck, and woodchuck models [14-19, 22, 33-35].

In this study, a DNA vaccine was produced and the CMI and humoral immune responses in chickens and expression of HA protein was measured. Results demonstrated that DNA-based immunization induced significant humoral and CMI immune responses with a T-helper 1 (Th1) preference.

Materials and methods

Virus, killed vaccine, anti-H1N1 chicken serum and cells

Avian influenza virus subtype H1N1 (A/blue-winged teal/AL/167/2007) was isolated at Auburn University [20]. This virus was passed four times in specific pathogen free (SPF) chicken embryonated eggs and adapted to grow on chicken embryo fibroblast cell (CEF). It's $TCID_{50}$ was $10^{8.5}$/50 µl. Allantoic fluid, was collected and used for viral RNA extraction with a TRIzol RNA extraction kit (Invitrogen, Carlsbad, CA). The killed H1N1 AIV vaccine and anti-H1N1 chicken serum were provided by Lohmann Animal Health International (Winslow, ME). COS-7 cells were purchased from American tissue collection center (ATCC), and maintained in minimal essential medium (MEM) containing 2% fetal bovine serum (FBS).

Chickens

Fertilized SPF eggs were obtained from the Auburn University poultry farm. Chicks were kept in plexiglass Horsfall-Bauer isolation units maintained with filtered air under negative pressure. Birds were given corn-soybean diet produced at Auburn University and water *ad libitum*. Care and handling of chickens was according to the Auburn University's Institutional Animal Care and Use Committee.

Plasmids construction

The pcDNA6.2 vector was obtained from Invitrogen (Carlsbard, CA). Full-length HA coding sequence was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using sense primer Bm-HA-(TATTCGTCTCAGGGAGCAAAAGCAGGGG) and antisense primer Bm-NS-890R (ATATCGTCTCGTATTAGTAGAAACAAGGGTGTTTT). RT-PCR was carried out following the manufacturer's protocol (Invitrogen, Carlsbad, CA) using the following program; 50^0C for 30 min, followed by 34 cycles of 94^0C for 15 s, 55^0C for 30 s, 72^0C for 1 min each cycle, and one cycle of 10 min at 72^0C. The RT- PCR product was examined by 1% agarose gel electrophoresis and purified using a QIA quick PCR purification kit (Qiagen, Valencia, CA). A 1.8 kb amplified fragment was cloned into a pcDNA6.2 vector (Invitrogen, Carlsbad, CA). The construct was a 7.6 kb plasmid designated as pcDNA6.2-HA. The recombinant clones were selected and analyzed by restriction enzyme digestion and DNA sequencing. The recombinant DNA vaccine vectors were purified using the Qiagen Endofree Plasmid Giga kit (Qiagen, Valencia, CA) and diluted in PBS used for transient transfection and immunization experiments.

In vitro and in vivo expression of the constructed plasmids.

For the transfection experiments, endofree plasmid DNA was used to transfect COS-7 cells using the ExGen 500 *in vitro* transfection reagent from Fermentas Inc. (Hanover, MD). COS-7 cells (100,000) were grown in a 12-well plate to 50-70% confluency in MEM containing 10% fetal bovine serum. Cells were transfected with 100 µl of a solution containing plasmid DNA-Transfection mixture in media. The transfection was carried out for 10 min and cells were incubated at 37^0C under 5% CO_2. The

expression of the AIV HN gene was evaluated by indirect immunofluorescence after 24 h using anti-AIV poly sera (Lohmann, Winslow, ME) for 1 hr. Goat-anti-chicken IgG conjugated with FITC (Southern Biotech, Birmingham, AL) was used as the secondary antibody. The HA expression was observed using an Olympus IX51 immunofluorescence microscope at 10X.

For *in vivo expression*, 50 µg of Endofree DNA vaccine vectors were injected IM into the thigh muscles of 7, 3-week-old chickens using a 1 µl syringe with 26-3/8 gauge needle. The muscles of three chickens were collected at 4 weeks after injection. The muscle was preserved in 30% sucrose made in phosphate buffered saline (PBS). Individual slides were evaluated by indirect immunofluorescence analysis. Briefly, tissues were fixed for 10 min with cold acetone and dried for 3 min. Slides were washed 3 times in 1X PBS, blocked with 0.1% FBS in PBS for 30 min, washed 3 times in 1X PBS, followed by addition of primary antibodies (anti-AIV chicken poly sera) without dilution, and incubated overnight at 4°C or at RT for 1hr. The wash steps were repeated and then diluted secondary antibodies (Goat anti-chicken IgG conjugated with FITC) at 1:500 dilutions in 0.1% FBS in PBS were added. Slides were incubated in the dark room at RT for 1-4 hr, washed and observed with a fluorescent microscope.

Vaccination of chickens

One-week-old SPF chickens were randomly separated into 5 groups (10/group). Group 1 and group 2 birds were controls and IM injected with 0.1 ml of saline and 25 µg of pcDNA6.2 DNA respectively; group 3 birds were given pcDNA6.2-HA DNA 25 µg at 1 week of age intramuscularly; group 4 birds were given pcDNA6.2-HA DNA 50 µg at 1 week of age intramuscularly. Chickens in group 1 to 4 were all treated at weekly intervals for three consecutive week; group 5 birds were IM injected with 1 dose of inactivated commercial H1N1 vaccine. Sera were collected from the wing vein of all birds on days 0, 14, 28, and 49. Serum samples were stored at -20^{0}C until they were analyzed. Spleens from 3 chickens per group were collected at day 49 for cytokine analysis. The remaining 7 chickens were sacrificed and thigh muscles were collected for immunohistology analysis.

Hemagglutination inhibition (HAI)

For determination of hemagglutination inhibition (HAI) titers, serum samples had been heat inactivated at 56^{0}C for 30 min prior to testing. Hemagglutination units (HAU) of the H1N1 AIV were determined before each assay using two-fold dilutions. Sera were serially diluted twofold in 25 µl PBS, and 4 HAUs of H1N1 were used in 25 µl. The contents of each well were gently mixed with a micropipettor and plates were incubated for 30 min at room temperature. Finally, 50 µl of a 0.5% chicken erythrocyte suspension was added to each well. The highest serum dilution capable of preventing hemagglutination was scored as the HAI titer. The HAI titer is directly correlated with immunity to AIV challenge. Data are reported a geometric means with standard deviation from three independent replicate experiments.

Virus neutralization assay

Sera were analyzed for AIV-specific neutralization titers according to a previous report [21]. Briefly, serum or a monoclonal antibody, which had been heat inactivated at 56^{0}C for 30 min, were added to the duplicate wells, and serial dilutions performed in the microtiter plates. All dilutions were made using MEM plus 2% fetal bovine serum (FBS), and the final volume was 75 µl per well. Chicken embryo fibroblasts (CEFs) were prepared from 9–11-day-old chicken embryos and grown in MEM containing 10 % FBS. 100 PFUs of AIV in 25 µl were added to each well, and the mixture incubated at 4 ^{0}C for 2 hr, following which, approximately 15,000 CEF cells in 100 µl of MEM plus 5% FBS were added to each well. Plates were placed at 37^{0}C in a CO_2 incubator for 3 days. The plates were fixed by aspirating the contents of the wells, washing three times with PBS at pH 7.2 with 0.5% Tween 20, adding

Table 1. Sequence of the primers used in qRT-PCR

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Accession no.
GAPDH[a]	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCCACA	K01458
IFN-γ[b]	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC	Y07922
IL-2[c]	TCTGGGACCACTGTATGCTCT	ACACCAGTGGGAAACAGTATCA	AF000631
IL-4[d]	ACCCAGGGCATCCAGAAG	CAGTGCCGGCAAGAAGTT	AJ621735
IL-6[e]	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTTCT	AJ309540
IL-10[f]	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC	AJ621614

[a] is the internal control
[b] and [c] are the main Th1 cytokines [30]
[d, e] and [f] are the main Th2 cytokines[31]

75 µl of an 80% (vol/vol) solution of acetone-PBS, and incubating at 4^0C for 15 min. After incubation, the contents were aspirated, and the plates were air dried.

Enzyme linked specific immunoassay (ELISA) was performed on the same plates with chicken anti-AIV poly antibody (1:500 dilution), and Goat-anti-Chicken Ig-HRP (1:2000 dilution). OD values were read at 450 nm. The AIV-specific percentage neutralization titer was defined as follows: % AIV neutralization titer = (1-sample $O.D._{450}$/RSV control $O.D._{450}$) X 100%. The neutralization assay was performed in triplicate and data expressed as the means of two determinations. Difference between groups were analyzed by variance (ANOVA) using SigmaStat statistical analysis software (Systat Software, San Jose, CA). Statistical significance was set at $P<0.05$.

Reverse transcription and quantitative PCR (qRT-PCR) for cytokine analysis

Total RNA was extracted from chicken spleen using TRIzol (Invitrogen, Carlsbad, CA) as described in manual. 5 µg of total RNA were treated with 1.0 unit of DNase I and 1.0 µl of 10x reaction buffer (Sigma, St. Louis, MO), incubated for 15 min at room temperature, 1.0 µl of stop solution was added to inactivated DNase I, and the mixture was heated at 70^0C for 10 min. Total RNA was reverse transcribed using Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations.

Oligonucleotide primers for chicken cytokines and GAPDH control were designed based upon sequences available from public databases (Table 1). Amplification and detection were carried out using equivalent amounts of total RNA from chicken spleen using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA).

QPCR data analysis

The relative transcriptional levels of different genes were determined by subtracting the cycle threshold (Ct) of the sample by that of the GAPDH, the calibrator or internal control, as per the formula: DCt = Ct (sample) - Ct (calibrator). The relative expression level of the specific gene in pcDNA6.2-HA vaccinated chicken compared to that in non-vaccinated chicken was calculated using the formula 2DDCt where DDCt = DCt (vaccinated) - DCt (non-vaccinated). Each analysis was performed in triplicate. Data were analyzed by analysis of variance (ANOVA) using SigmaStat statistical analysis software (Systat Software, San Jose, CA). Statistical significance was set at $P<0.05$.

Results

Construction and identification of the in vivo and in vitro expression of the plasmid containing AIV HA gene

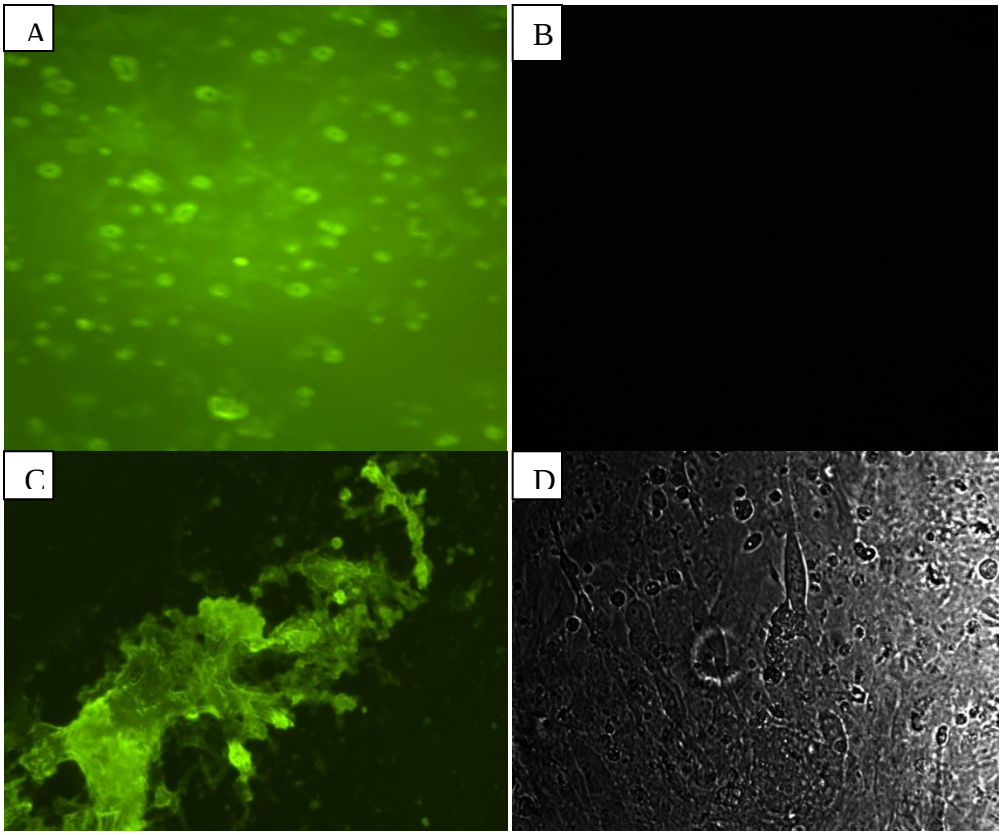


Figure 1. *In vitro* and *in vivo* expression of the encoded HA protein. COS-7 cells were transfected with the plasmid pcDNA6.2-HA by ExGen 500. Expression of the encoded protein was detected at 48 hr after transfection (A). COS-7 cells transfected with pcDNA6.2 only (B). 3-weeks-old SPF chickens were IM injected with the plasmid pcDNA6.2-HA. Breast muscles were collected 4 weeks after injection. Protein expression was detected by FA assay with a polyclonal antibody (C). Muscle tissue taken from untreated chicken (D).

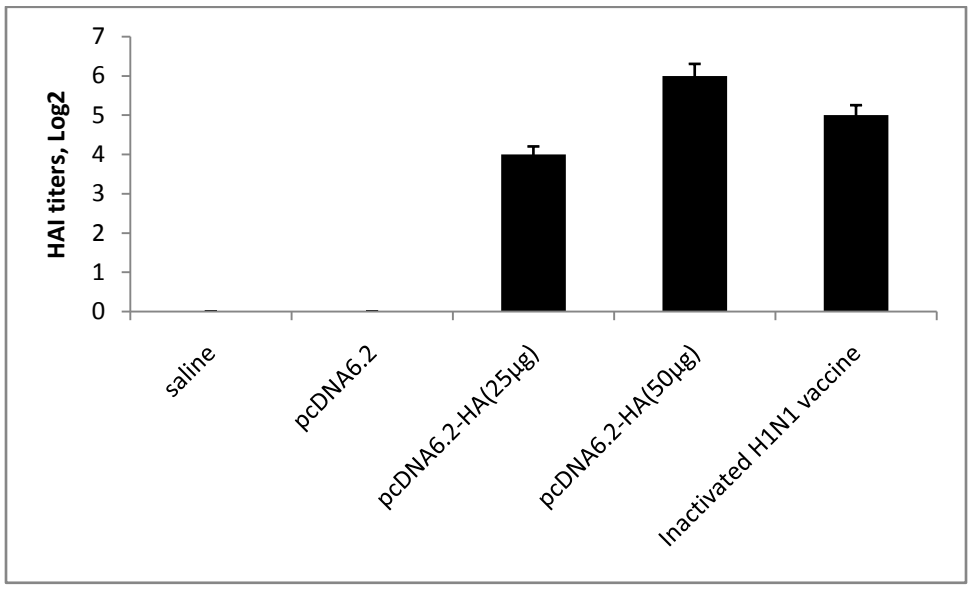


Figure2. Specific antibody titers in chickens. Data represent the mean (N=10) ± S.D for titers of anti-AIV antibodies as determined in triplicate by HI.

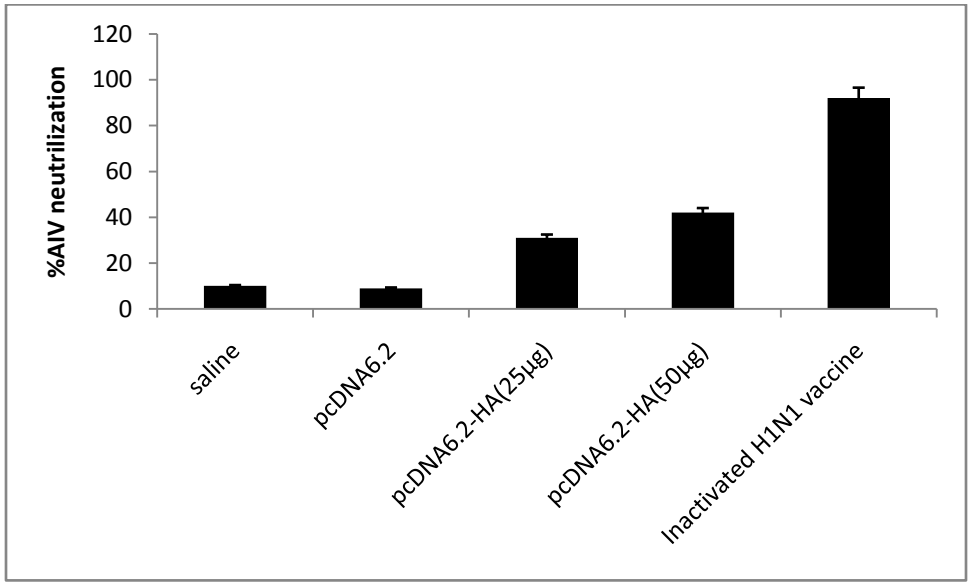


Figure 3. % Neutralization antibody response. Results are presented as an average from three groups in triplicate; error bars represent standard deviation.

In vivo expression of the HA gene in COS cells detected by indirect immunofluorescence is listed in Figures 1A and 1B. Protein expression in thigh muscles listed in Figures 1C and 1D.

Virus-specific antibody responses

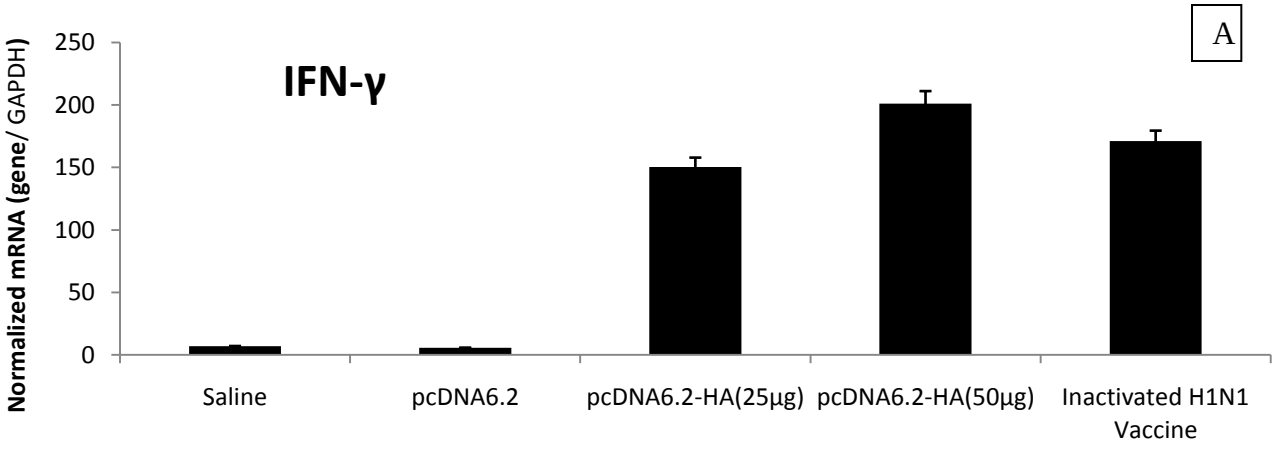
Chickens in the group, which received 50 µg of pcDNA-HA IM injection had the highest serum antibodies. The titer was significantly higher than the titers of chickens in the saline, plasmid control group of commercial vaccinated groups (Figure 2). Substantial neutralization (VN) assay confirmed these antibodies (Figure 3) had strong VN activity. Chickens, which received a high dose of plasmid (50 µg) DNA induced higher VNs compared to the chickens, which received the low dose (25 µg) plasmid DNA. However, the titers were lower than the birds which received the commercial vaccine.

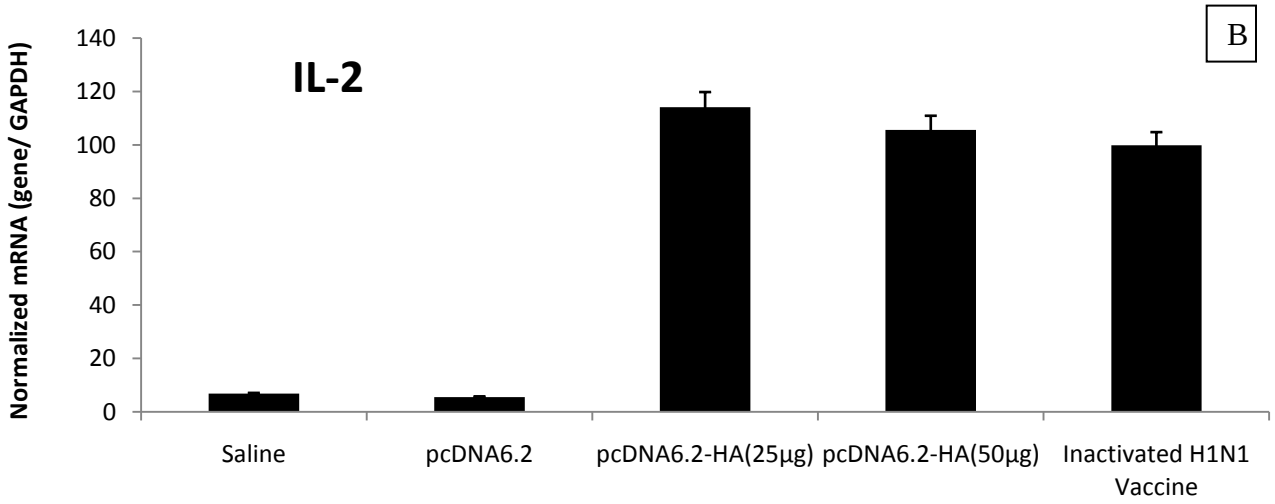
Cytokine analysis

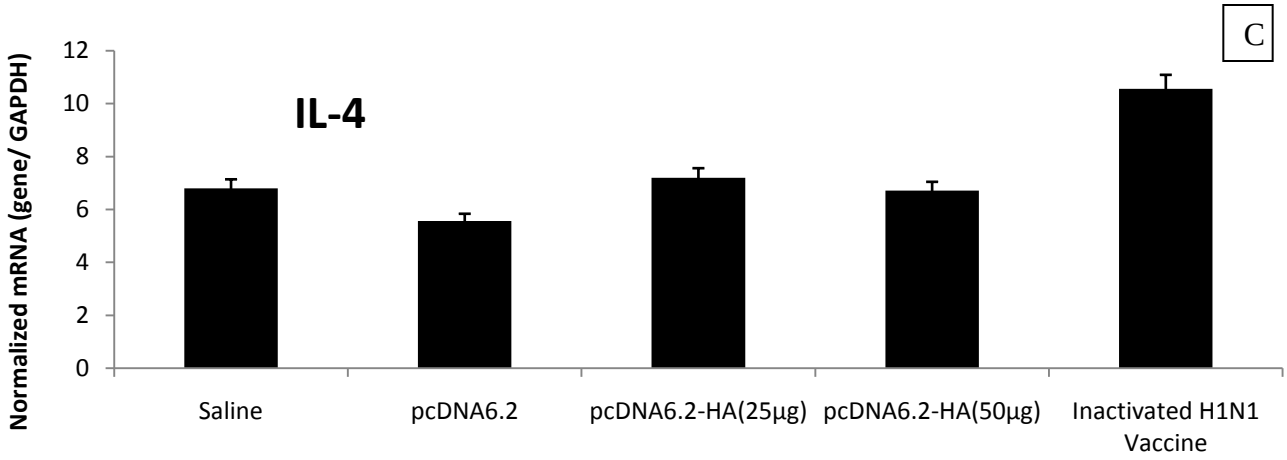
The subsets of Th cells were distinguished by the pattern of cytokines. To distinguish between the activation of Th cells of the Th1 and Th2 subsets, the expression levels of mRNA encoding a panel of chicken cytokines were quantified in spleen lymphocyte following DNA vaccine inoculation. Compared with negative controls, transcripts of the cytokines IFN-γ, IL-2 were increased up to 50-fold following third inoculation in chickens, which received the high dose of plasmid DNA (50 µg/time). This was a significant increase of Th1 cytokine production (Figure 4A & 4B). The production of Th2 cytokine IL-4 was not significantly different in any of the groups (Figure 4C). The production of Th2 cytokines IL-6 and IL-10 was not significantly different from group 1 to group 4 ($P<0.05$), but activated vaccine (group 5) produced a high titer of IL-6 and IL-10 (4D & 4E).

Discussion

LP AIVs can produce slight respiratory reactions in poultry and can infect and recombine with IVs from other species resulting in HP IVs. Control measures must be instituted to prevent the spread of AIVs (LP or HP) between species. Currently most conventional vaccines for the control and prevention of AIV outbreaks are focused on HP H5 and H7 AIVs. However, there is a need to develop vaccines and measure the humoral and CMI responses against LP AIVs. The 2009 North American swine flu virus pandemic contained AIV genes of unknown pathogenicity. The HA gene of the H1N1 AIV


A
IFN-γ
Normalized mRNA (gene/ GAPDH)
250
200
150
100
50
0
Saline
pcDNA6.2
pcDNA6.2-HA(25μg)
pcDNA6.2-HA(50μg)
Inactivated H1N1 Vaccine


B
IL-2
Normalized mRNA (gene/ GAPDH)
140
120
100
80
60
40
20
0
Saline
pcDNA6.2
pcDNA6.2-HA(25μg)
pcDNA6.2-HA(50μg)
Inactivated H1N1 Vaccine


C
IL-4
Normalized mRNA (gene/ GAPDH)
12
10
8
6
4
2
0
Saline
pcDNA6.2
pcDNA6.2-HA(25μg)
pcDNA6.2-HA(50μg)
Inactivated H1N1 Vaccine

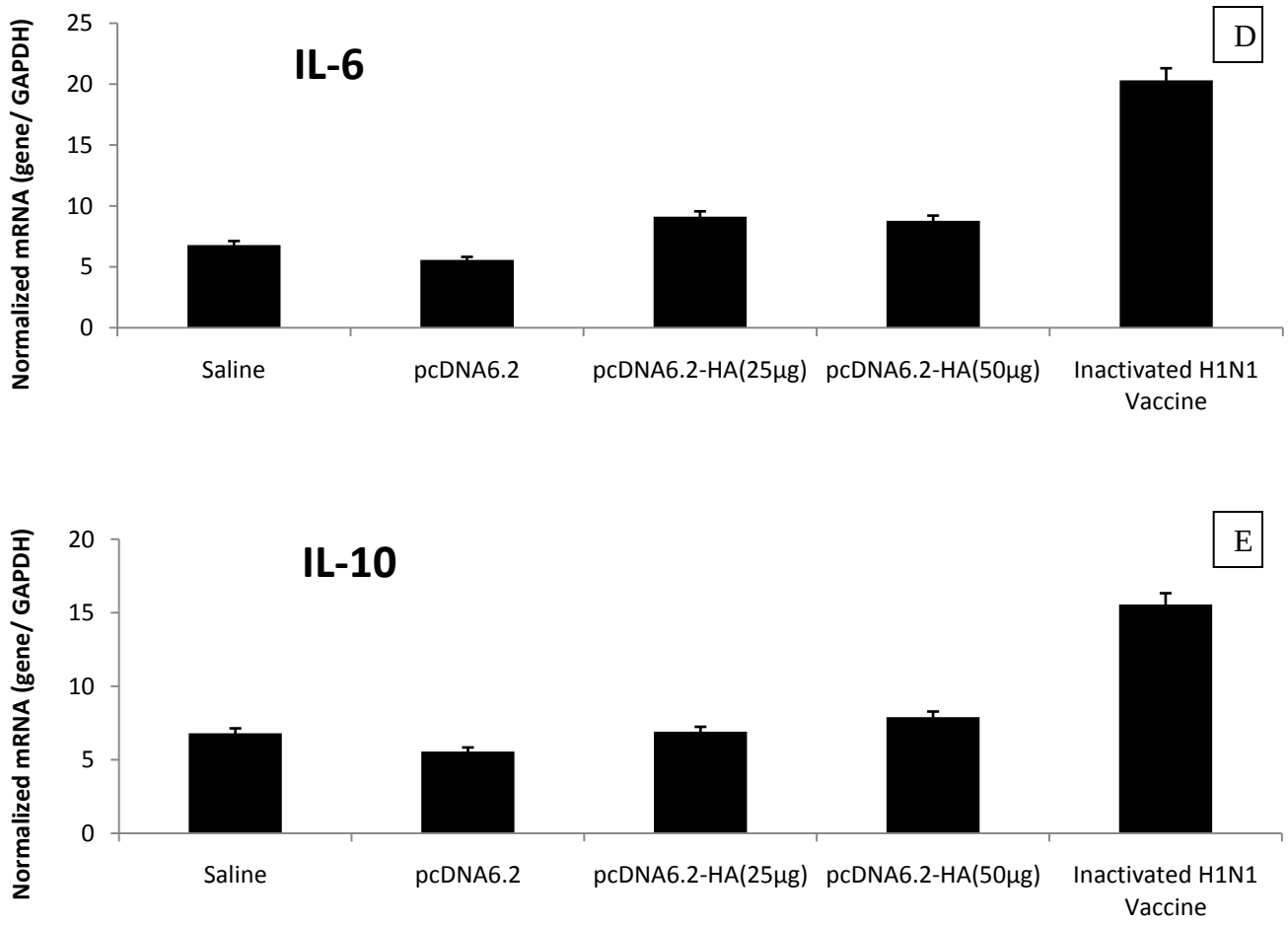


Figure 4. Effect of plasmid on AIV-specific Th1/Th2 levels in immunized chickens. Chickens in group 1 and 2 were controls IM injected with 0.1 ml of saline and 25 µg of pcDNA6.2 DNA respectively; group 3 birds were given pcDNA6.2-HA DNA 25 µg at 1 week of age by IM route; group 4 birds were given pcDNA-HA DNA 50µg IM at 1week of age. Groups 1 to 4 were all treated at weekly interval for three consecutive weeks; group 5 birds were IM injected with 1 dose of inactivated H1N1 vaccine. One week after the third immunization, the expression levels of mRNA in spleenocytes encoding chicken cytokines were quantified with real-time RT-PCR.

from Alabama was 95% identical with the virus isolated from swine isolated in Canada [20]. Based on the recent H1N1 pandemic influenza experience, there is need to develop a vaccine to prevent the spread of this and other AIVs between birds, swine, and humans.

DNA vaccination has gained considerable importance since its discovery in the early 1990s [23]. Studies showed that DNA vaccines are effective in the induction of humoral and/or CMI and confers protection against various agents [23, 24, 25, 26]. Our ultimate goal is to develop a DNA-mediated vaccination to protect chickens against infection and disease with HP or LP pathogenic AIVs. The HA gene of AIV has been successfully used as killed subunit or recombinant viral vector based vaccines against HP H5 or H7 [26, 27]. However, protection against LP AIVS using DNA-mediated vaccination has received little attention.

Studies have shown that CMI can provide protection from the morbidity and mortality associated with HP IV infections [28]. In this present experiment the DNA vaccine containing a LP H1N1 AIV isolated from local ducks

containing plasmid vectors encoding HA induced significant humoral immune and CMI responses. The HI, but not VN antibody, elicited by the DNA vaccine was higher than the commercial killed vaccine and significantly better than a plant-derived AIV vaccine [29]. But HI antibody is not absolutely proportional to protection, the VN antibody should be more important indicating vaccine efficacy [35]. Our result suggests killed vaccine have better protection than DNA vaccine; our future challenge test will further confirm this assumption. Moreover, it was known that Th1 cells produce IFN-γ and IL-2, which play a critical role in determining CMI responses. CMI is important for the clearance of intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-10, and IL-13. They are associated with allergies and humoral responses [30, 31]. DNA vaccines, which could induce Th1 immune responses, might provide prevention for AIV infections and diseases. The experiment agrees with recent research on a DNA-based prime-boost strategy, which showed that the experimental DNA based vaccine produced significant IFN-γ, IL-2, IL-5, and IL-10 [32]. This is important since challenge with LP viruses in chickens does not produce consistent measurable results and the prevention of the spread of LP viruses such as the H1N1 to birds, swine, or human by vaccination may prevent recombination thereby preventing a pandemic. Also, this approach is more cost effective and does not depend upon the use of high pathogenic AIV strains and need for a BSL3 facility, furthermore we will measure virus shedding in the future challenge test, oropharyngeal and cloacal swabs will be obtained from all chickens on days 4, 6, and 8 after challenge, the virus titer will represent the protection.

In conclusion, results presented here showed that IM DNA-based immunization in chickens produced a moderate anti-AIV HI response and a low anti-AIV VN response, when compared to the commercial vaccine. It also induced CMI related cytokines in splenocytes. Cytokine mRNA levels after the third immunizations were highly up-regulated, with a Th1 preference.

Acknowledgements

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