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## Strategies for improving the efficacy of a H6 subtype avian influenza DNA vaccine in chickens

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### Abstract

A low-pathogenicity avian influenza H6N2 virus was used to investigate approaches to improve DNA vaccine efficacy. The viral hemagglutinin (HA) gene or its chicken biased HA gene, incorporating a Kozak sequence, was cloned into a pCAGGS vector to produce the pCAG-HA<sub>k</sub> and pCAG-optiHA<sub>k</sub> constructs. Following two intramuscular injections, the seroconversion rate in vaccinated chickens with 10, 100 or 300 µg pCAG-HA<sub>k</sub> were 87.5%, 75% and 75% respectively. The profile of H6 hemagglutination inhibition (HI) antibodies induced by different doses of pCAG-HA<sub>k</sub> during the 8-week study period was similar. The HI titer rose significantly in the three different dose groups following the booster and reached a plateau 2-3 weeks post-booster. In a single dose vaccination group with 100 µg pCAG-HA<sub>k</sub>, a maximum seroconversion rate reached 53.3% at 5 weeks post-vaccination. The earliest time of seroconversion appeared two weeks after DNA immunization. Following two electroporation (EP) vaccinations with 100 µg pCAG-HA<sub>k</sub>, all birds seroconverted and the HI antibody titers were significantly higher than those using intramuscular immunization, suggesting that EP was more efficient than intramuscular delivery of the DNA vaccines. In comparison, chickens immunized with 10 or 100 µg pCAG-optiHA<sub>k</sub> showed 37.5% and 87.5% seroconversion rates respectively at 3 weeks following the booster. The pCAG-HA<sub>k</sub> was not significantly different from the pCAG-optiHA<sub>k</sub> in either the seroconversion rate or H6 HI titer,

suggesting that the codon-optimized HA DNA vaccine did not achieve significantly better immunogenicity than the pCAG-HA<sub>k</sub> vaccine.

*Keywords:* Avian influenza; H6 subtype; DNA vaccine; Codon optimization; Electroporation; Chickens

## **1. Introduction**

DNA vaccines have been developed to protect against a number of pathogens in a range of animals following the first report that direct injection of plasmid DNA generated the successful expression of the plasmid-encoded antigen in murine muscle cells (Wolff et al., 1990). However, low and variable antibody response has been a critical problem hindering commercial DNA vaccine development (Abdulhaqq and Weiner 2008). In response, a diverse range of strategies have been developed to address the factors that limit immune responses to DNA vaccines. However, little data are available to indicate which of these strategies will be most useful and practical.

Successful DNA vaccination requires high expression of genes derived from microorganisms in animals and humans. The Kozak sequence plays a major role in the initiation of a translation process in mammalian cells by slowing down the rate of scanning by the ribosome and increasing the chance of the ribosome recognizing the start of translation at the AUG start codon (Kozak 1987). Insertion of a Kozak sequence into a gene of interest or into an expression vector has been used to develop DNA vaccines against Chikungunya virus (Muthumani et al., 2008) and HIV (Kumar et al., 2006), and for minigene DNA vaccines (An et al., 2000).

It is known that mammalian codon usage patterns are different from those of numerous microorganisms and yeasts (Ikemura 1985). Thus, differences in codon usage between a heterologous gene and the host organism may have an impact on expression of the gene,

which may eventually affect the immunogenicity of an antigen-encoding DNA vaccine. It was reported that codon optimization was effective in enhancing the immunogenicity of DNA vaccines against influenza virus (Wang et al., 2006), H5 AIV (Jiang et al., 2007), HIV (Andre et al., 1998) and *Mycobacterium tuberculosis* (Ko et al., 2005).

Intramuscular (IM) vaccination has been used widely, however, it does not appear particularly efficient at inducing immune responses by DNA vaccination, as plasmid DNA administered by IM route was poorly distributed, inefficiently expressed, and rapidly degraded (Wang et al., 2008). Physical methods, such as a gene gun and electroporation (EP), have been shown to elicit better immune responses in large animals and humans (Wang et al., 2008). Although the gene gun technology was regarded as an effective method for DNA vaccination, this method is currently impractical for poultry due to the inability to vaccinate large numbers of birds and the high cost of the carrier beads (Kodihalli et al., 1997). *In vivo* electroporation has been demonstrated to improve the efficacy of DNA vaccination in eliciting both humoral and cellular immune responses in mice, rats, guinea pigs and rabbits (Widera et al., 2000; Zucchelli et al., 2000).

DNA vaccines encoding HA genes of influenza type A viruses have been reported to generate variable immune responses in several different species, including mice (Montgomery et al., 1993), chickens (Kodihalli et al., 1997), swine (Macklin et al., 1998), ferrets (Ljungberg et al., 2002) and nonhuman primates (Liu et al., 1997). In our previous experiments, IM injection of four DNA constructs encoding the same HA gene of an avian influenza H6N2 virus in different expression vectors (VR1012, pCI, pCI-neo and pVAX1) induced no or marginal antibody response in chickens (Shan 2010). In this study strategies were investigated to improve the immune responses to a DNA vaccine

encoding the HA gene of a H6N2 low pathogenicity avian influenza (LPAI) virus in a pCAGGS vector in chickens. The strategies investigated with this vector included insertion of a Kozak sequence, use of a chicken codon optimized HA gene and use of EP, in an attempt to improve the efficacy of DNA vaccines.

## **2. Materials and methods**

### *2.1. Animals, viruses and cells*

The LPAI virus used for these studies, A/Eurasian coot/Western Australia/2727/79 (A/coot/WA/2727/79) (H6N2), was propagated and titrated as 50% egg infectious doses/0.1 mL (EID<sub>50</sub>/0.1 mL) as described previously (Shan et al., 2010).

Three-week-old SPF chickens were housed, fed and watered in high efficiency particulate air (HEPA)-filtered poultry isolators according to the experimental protocol approved by the Animal Ethics Committee of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China.

*In vitro* expression of the DNA vaccine construct was conducted in a human embryonic kidney cell line, 293T cells, and also in primary cultures of chicken embryo fibroblast (CEF) cells prepared as described previously (Shan, 2010). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin at pH 7.2 and 37 °C in a 5% carbon dioxide atmosphere.

### *2.2. Construction of expression plasmid (pCAG-HA<sub>k</sub> and pCAG-optiHA<sub>k</sub>)*

Amplification of the full-length open reading frame (ORF) of the HA of A/coot/WA/2727/79 (H6N2) was performed using the primers

5'ATGATTGCAATCATAATATTC3' and 5'TTATATACATATCCTGCATTG3'. A Kozak sequence (GCCGCCACC) was inserted before the ATG start codon, an *EcoRI* site was added upstream of the Kozak sequence and a *SmaI* site was added to the 5' end of the downstream primer. The primers were synthesized by GeneWorks (GeneWorks Pty. Ltd., Australia). The HA gene extracted from virus infected allantoic fluid was amplified by proof-reading PCR using the Phusion<sup>TM</sup> high-fidelity DNA polymerase protocol (Finnzymes OY, Finland) according to the manufacturer's instructions, and was subsequently cloned into the plasmid vector pCAGGS (kindly provided by Dr. Y. Kawaoka) to generate the DNA construct pCAG-HA<sub>K</sub>. The pCAGGS plasmid is a monocistronic vector which contains the chicken  $\beta$ -actin promoter and the human CMV-IE enhancer, the SV40 origin of replication and the rabbit  $\beta$ -globulin polyadenylation signal.

The sequence of the full-length HA gene from A/coot/WA/2727/79 (H6N2) was shifted to the chicken-biased codons using DNASTAR from a codon usage database (<http://www.kazusa.or.jp/codon/>). The Kozak sequence and restriction endonuclease site sequences for *EcoRI* and *SmaI* were included at the appropriate locations as described above, and this codon-optimized HA gene was chemically synthesized by GenScript Corporation (Beijing, China). The synthesized codon-optimized HA gene insert was subcloned into the pCAGGS vector to generate the DNA construct pCAG-optiHA<sub>K</sub>.

Plasmids pCAG-HA<sub>K</sub>, pCAG-optiHA<sub>K</sub> and pCAGGS were amplified in *E.coli* DH5 $\alpha$  (TaKaRa, China) and extracted using the alkaline lysis method (Sambrook and Russell 2001). After purification by polyethylene glycol (PEG8000) precipitation, the plasmids

were quantified by ultraviolet spectrophotometer (Ultrospec3000, Pharmacia Biotech Inc., Sweden) and kept at  $-20^{\circ}\text{C}$  before the animal studies.

### *2.3 In vitro expression of DNA vaccines*

Transfection of pCAG-HA<sub>k</sub> and pCAG-optiHA<sub>k</sub> into 293T and CEF cells was conducted with Lipofectamine<sup>TM</sup> 2000 (Invitrogen, USA) following the manufacturer's instructions. Forty eight hours after transfection, the cells were fixed with 75% ethanol for 30 min at room temperature. An immunofluorescence assay was performed using chicken anti-H6 serum and FITC-conjugated rabbit anti-chicken IgG (Sigma-Aldrich, China). The monolayers were observed for specific green fluorescence under a Leica DMIRES2 microscope (Leica, Germany).

### *2.4 Animals, immunizations and antibody responses*

Vaccine trial experiments in SPF chickens were conducted in HEPA-filtered isolators. The plasmids were diluted in PBS to the desired concentration and administered, as indicated in Table 2 and 3, in a 200  $\mu\text{l}$  volume to the leg muscle by either IM injection or EP. Three groups of 8 chickens each received either 10, 100 or 300  $\mu\text{g}$  pCAG-HA<sub>k</sub> by IM injection (Groups 1-3); one group of 8 chickens received 100  $\mu\text{g}$  pCAGGS by IM injection (Group 4) and one group of 8 chickens received 100  $\mu\text{g}$  pCAG-HA<sub>k</sub> by EP (Group 5). Three weeks later, these five groups of birds were given booster vaccinations using the same dose and delivery route. Another group of 15 chickens (Group 6) received only a single vaccination of 100  $\mu\text{g}$  pCAG-HA<sub>k</sub> by IM injection. Two other groups of 8 chickens each received either 10 or 100  $\mu\text{g}$  pCAG-optiHA<sub>k</sub> by IM injection followed three weeks later by booster vaccinations using the same dose and delivery route (Groups 7 and 8).



Blood was collected weekly from the wing vein of each chicken for testing of serum antibody levels to H6 HA. Antibody was measured using the haemagglutination inhibition (HI) test as described previously with A/coot/WA/2727/79 (H6N2) virus as antigen (Shan, 2010).

### *2.5 Data analysis*

Statistical analysis of HI antibody titers was conducted as geometric mean titers (GMT). Statistical analyses of experimental data were conducted by using one way analysis of variance (ANOVA) with SPSS statistical software version 15 as well as Chi-square (Fisher's exact test was used when the sample number was less than 5) using Statistix. Statistical significance was defined at the level of  $p < 0.05$ .

## **3. Results**

### *3.1 Effect of codon optimization on HA composition*

The chemically synthesized chicken codon-biased HA gene from A/ coot/WA/2727/79 (H6N2) had the same HA amino acid sequences as the original virus, but the nucleotide homology between the wild-type HA gene and the codon-optimized HA gene was 74.8% (The nucleotide sequence comparison using ClustalW tool between them is not shown, but is available on request). A summary of the codon preference changes before and after codon optimization using EditSeq of DNASTAR is presented in Table 1. Codon optimization resulted in an increase in the G+C content from 41.3% in HA wild-type to 59.5% after optimization.

### *3.2 In vitro expression of the pCAG-HAk and pCAG-optiHAk constructs*

After cloning of the HA or codon optimized HA gene into the pCAGGS vector, the positive clones were screened by PCR and the resultant pCAG-HA<sub>k</sub> or pCAG-optiHA<sub>k</sub> construct was confirmed by restriction enzyme analysis and sequencing. Expression of influenza H6 HA by the plasmids was confirmed by immunostaining the transfected 293T (shown in Fig. 1) and CEF cells. The pCAG-HA<sub>k</sub> or pCAG-optiHA<sub>k</sub> constructs showed similar intensity of fluorescence in either 293T or CEF cells.

Table 1 Comparison of codon preference before and after codon optimization.

Amino acid	Codon		Amino acid	Codon	
	Original	Codon optimization		Original	Codon optimization
Ala	GCA	GCC	Met	AUG	AUG
Arg	AGA	AGA	Leu	UUG	CUG
Asn	AAU	AAC	Lys	AAA	AAG
Asp	GAU	GAU	Phe	UUC/UUU	UUC
Cys	UGU	UGC	Pro	CCA	CCC
Gln	CAA	CAG	Ser	UCA	AGC
Glu	GAA	GAG	Thr	ACA	ACC
Gly	GGA	GGC	Trp	UGG	UGG
His	CAU	CAC	Tyr	UAU	UAC
Ile	AUA	AUC	Val	GUG	GUG

### 3.3 Antibody response induced by two vaccinations of pCAG-HA<sub>k</sub> construct

As shown in Table 2 (Groups 1 to 3), the earliest time of seroconversion appeared two weeks after vaccination. The H6 HI test seroconversion rates three weeks after initial vaccination were 37.5%, 12.5% and 0 in the 10, 100 and 300 µg pCAG-HA<sub>k</sub> vaccinated groups respectively. After the booster vaccination the seroconversion rate increased to 87.5%, 75% and 75% respectively, indicating that a second vaccination increased the seroconversion rate in all three groups.

The profile of HI antibodies induced by different doses of pCAG-HA<sub>k</sub> (Groups 1 to 3) during the 8-week study period was similar (Table 3). After one dose, the vaccinated birds in the 10 µg and 100 µg pCAG-HA<sub>k</sub> groups demonstrated HI antibody, but those in

the 300 µg group did not. However, the H6 HI titer rose significantly in the three different dose groups following booster vaccination and reached a plateau 2-3 weeks post-booster. No H6 HI antibody was detected in the pCAGGS vaccinated group. The GMT of HI antibody was significantly different (Mann-Whitney Test,  $p < 0.05$ ) between the combined pCAG-HA<sub>k</sub> groups and the pCAGGS group at weeks 4, 5, 6, 7 and 8 following initial vaccination, but there was no significant difference at week 3 after vaccination. There was no significant difference in GMT between the three doses of pCAG-HA<sub>k</sub> vaccine (ANOVA,  $P = 0.963$ ).

#### *3.4 Antibody response induced by a single vaccination of the pCAG-HA<sub>k</sub> construct*

Of 15 birds vaccinated with a single dose of 100 µg pCAG-HA<sub>k</sub> construct, 40% (6/15) seroconverted at week 2 post-vaccination and 66.7% (10/15) at week 5 post-vaccination (Group 6 in Table 2). The peak HI GMT occurred at week 5 post-vaccination and the HI titer slowly declined during the 3-month observation period. There were no significant differences (ANOVA,  $P > 0.05$ ) in the GMT of H6 HI antibody at weeks 2 and 3 post-vaccination between Group 6 and the pCAGGS vector group (Group 4). Neither was there a significant difference in the HI antibody (ANOVA,  $P = 0.43$ ) or in the seroconversion rate (Fisher's exact test,  $P = 0.37$ ) at week 3 following initial vaccination between Group 6 and Group 2 (Table 2 and Table 3).

#### *3.5 Antibody response induced by two vaccinations of pCAG-HA<sub>k</sub> construct via EP*

Birds administered pCAG-HA<sub>k</sub> construct via EP showed 87.5% seroconversion after the first vaccination and 100% seroconversion after the second vaccination (Group 5 in Table 2 and Fig. 2). The highest HI antibody GMT ( $2^{7.1}$ ) occurred at week 5 post-vaccination, and then the antibody levels gradually declined. The highest HI titer for an individual

bird was  $2^{11}$ . There were significant differences (ANOVA/Mann-Whitney Test,  $P < 0.05$ ) in the HI antibody titers from week 2 to 8 between the IM and EP groups receiving 100  $\mu\text{g}$  pCAG-HA<sub>k</sub>.

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Table 2 Seroconversion rate by H6 HI test in chickens immunized with pCAG-HAk, pCAG-optiHak or pCAGGS.

Group	Vaccine	Dose (µg)	Route	Seroconversion rate (weeks post vaccination)											
				1	2	3 <sup>a</sup>	4	5	6	7	8	9	10	11	12
1	pCAG-HAk	10	IM	0/8 <sup>bA</sup>	1/8 <sup>A</sup>	3/8 <sup>A</sup>	4/8 <sup>A</sup>	6/8 <sup>A</sup>	6/8 <sup>A</sup>	7/8 <sup>A</sup>	7/8 <sup>B</sup>				
2	pCAG-HAk	100	IM	0/8 <sup>A</sup>	1/8 <sup>A</sup>	1/8 <sup>A</sup>	2/8 <sup>A</sup>	6/8 <sup>A</sup>	5/8 <sup>A</sup>	5/8 <sup>A</sup>	6/8 <sup>AB</sup>				
3	pCAG-HAk	300	IM	0/8	0/8	0/8	5/8	6/8	6/8	6/8	5/8				
4	pCAGGS	100	IM	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8				
5	pCAG-HAk	100	EP	1/8	5/8	7/8	8/8	8/8	8/8	8/8	8/8				
6	pCAG-HAk	100	IM	0/15	6/15	5/15	7/15	10/15	8/15	8/15	8/15	8/15	8/15	8/15	8/15
7	pCAG-optiHak	10	IM	0/8 <sup>A</sup>	0/8 <sup>A</sup>	1/8 <sup>A</sup>	1/8 <sup>A</sup>	2/8 <sup>A</sup>	3/8 <sup>A</sup>	3/8 <sup>A</sup>	2/8 <sup>A</sup>	2/8 <sup>A</sup>			
8	pCAG-optiHak	100	IM	0/8 <sup>A</sup>	2/8 <sup>A</sup>	3/8 <sup>A</sup>	3/8 <sup>A</sup>	4/8 <sup>A</sup>	4/8 <sup>A</sup>	7/8 <sup>A</sup>	7/8 <sup>B</sup>	7/8 <sup>B</sup>			

<sup>a</sup> Time of booster vaccination, except that group 6 was only vaccinated once.

<sup>b</sup>N/N represents HI antibody positive chickens/all vaccinated chickens. Within the column the different uppercase superscript letter indicates statistical differences ( $P < 0.05$ ) using Fisher's exact test, group 3 to 6 excluded in statistical analysis.

Table 3 HI titers in chickens immunized with pCAG-HAk, pCAG-optiHak or pCAGGS over an 8- or 12- week period after vaccination.

Vaccine	Dose (µg)	Route	HI titer (weeks post vaccination)												
			1	2	3	4	5	6	7	8	9	10	11	12	
pCAG-HAk	10	IM	0 <sup>a</sup>	0.4±1.1	0.9±1.4	1.6±2.1	2.4±2.1	2.8±2.1	3.1±2.0	3.2±2.1					
pCAG-HAk	100	IM	0	0.4±1.1	0.4±1.1	1.0±2.1	2.6±2.6	2.9±2.9	2.9±2.7	2.9±2.3					
pCAG-HAk	300	IM	0	0	0	1.5±1.7	2.6±2.2	2.6±2.0	2.3±1.7	1.9±1.8					
pCAGGS	100	IM	0	0	0	0	0	0	0	0					
pCAG-HAk	100	EP	0.1±0.4	3.3±3.4	3.8±2.8	6.7±3.0	7.1±3.0	6.6±2.3	5.8±2.4	5.6±2.1					
pCAG-HAk <sup>b</sup>	100	IM	0	1.0±1.4	0.9±1.4	2.0±2.5	2.4±2.3	2.1±2.2	1.8±1.9	1.7±1.8	1.5±1.6	1.3±1.3	1.1±1.2	1.1±1.2	
pCAG-optiHak	10	IM	0	0	0.1±0.2	0.4±1.1	1.3±2.5	1.4±2.5	1.1±2.0	1.2±2.2	1.2±2.2				
pCAG-optiHak	100	IM	0	1.1±1.5	0.8±1.3	1.4±1.9	2.5±2.7	2.6±2.8	3.2±2.3	2.9±2.2	3.0±2.1				

<sup>a</sup> H6 HI antibody GMT(log<sub>2</sub>± SD).

<sup>b</sup> This group was only vaccinated once. The others were given a booster 3 weeks after initial immunization.

### *3.6 Antibody response induced by two vaccinations of pCAG-optiHAk construct*

The kinetics of the antibody response induced by pCAG-optiHAk is shown in Table 2 and 3. Following booster vaccination, the seroconversion rate and HI titers increased in both groups. The higher dose groups generated better immune responses than the lower dose groups in terms of the seroconversion rate, although there were no significant differences in the GMT of HI antibody between different doses of pCAG-optiHAk. This suggested that a 100 µg dose of pCAG-optiHAk was a more optimal dosage for induction of HI antibody responses.

### *3.7 Comparison of HI titers between pCAG-HAk and pCAG-optiHAk groups*

The seroconversion rate and level of antibody response were compared between pCAG-HAk and pCAG-optiHAk groups (Tables 2 and 3). There were no significant differences in the seroconversion rates from week 1 to week 7 after initial vaccination between either 10 or 100 µg groups for pCAG-HAk and pCAG-optiHAk vaccines. Neither were there significant differences (ANOVA or Mann-Whitney,  $P > 0.05$ ) over an 8-week period in post vaccination antibody titers between pCAG-HAk and pCAG-optiHAk vaccines. Only at week 8 for the 10 µg dose groups was there a difference, with the pCAG-HAk group showing a higher seroconversion rate than the pCAG-optiHAk group.

## **4. Discussion**

This study demonstrated that a pCAGGS vector encoding HA or codon optimized HA of a H6N2 virus, together with insertion of a Kozak sequence, was able to elicit measurable H6 HI antibody responses in SPF chickens. Either one or two vaccinations of naked plasmid DNA induced seroconversion in a substantial proportion of vaccinated chickens. Although the seroconversion rate following two vaccinations was similar in the three

different dose groups, both 10  $\mu\text{g}$  and 100  $\mu\text{g}$  groups appeared to generate higher H6 HI geometric antibody titers than the 300  $\mu\text{g}$  group although this was not significantly different. This observation that lower dose DNA vaccination was equivalent to or better than higher dose DNA vaccination for generating antibody responses was comparable to a previous evaluation by Shan (2010) using four different HA-DNA vaccine constructs in chickens. In addition, vaccination with the 10  $\mu\text{g}$  pCAG-HAk DNA plasmid dose via the IM route appeared to induce similar H6 HI antibody titers to the 100  $\mu\text{g}$  DNA plasmid dose. In contrast, the 100  $\mu\text{g}$  dose pCAG-optiHAk group seemed to elicit a better response than the 10  $\mu\text{g}$  dose group. This underlined the critical need for optimization of DNA vaccine doses.

In the group receiving a single dose of the 100  $\mu\text{g}$  pCAG-HAk DNA vaccine, the antibody response at week 3 post-vaccination appeared stronger in terms of the sero-conversion rate than that in the group with two injections of the same dose 3 weeks apart. However, the differences between the groups at week 3 post-vaccination were not significantly different and probably resulted from biological variation in individuals within the experimental groups. In addition, there was considerable individual variation in H6 HI antibody response among chickens inoculated intramuscularly with pCAG-HAk vaccine, or pCAG-optiHAk vaccine, or even those vaccinated using EP. This variation has also been reported in other DNA vaccine studies in chickens (Suarez and Schultz-Cherry 2000) and mice (Ulmer et al., 1994). The reason for the wide variation in individual HI antibody titers in vaccinated birds is not clear, but may be attributable to the poor efficiency of the delivery method (Wang et al., 2008). This inter-individual

variation is another drawback of DNA vaccines and highlights the need to fully elucidate the mechanism of the DNA-induced immune response.

The temporal antibody response studies following pCAG-HA<sub>k</sub> vaccination with EP showed that the earliest detectable antibody was at one week post-vaccination, compared with two weeks via IM administration. However, in some IM vaccinated chickens the immune response was not detectable until 5 weeks post-vaccination. This long lag phase is probably related to poor DNA uptake into muscle fibers via IM delivery, the mechanism of which is poorly understood. It has been hypothesized that although IM injection of naked DNA vaccines only causes the transfection of a limited number of muscle fibers, the low rate of muscle fiber turnover can result in the expression of the plasmid DNA for an extended period of time (Pertmer et al., 1996). In addition, myocytes (long-lived cells that cannot re-enter mitosis) are terminally differentiated, thus they do not dilute out the internalized plasmids, resulting in long-term stability and transcriptional activity of plasmid DNA (Fattori et al., 2002).

It has been established previously, based on disease signs and tracheal/cloacal virus isolation, that chickens given inactivated vaccines reaching a post-vaccination geometric mean HI titer of greater than 6.5 log<sub>2</sub> had good protection against virus challenge (Wood et al., 1985). However, in vaccine challenge studies using field vaccinated birds with a H5 HI titer of 5.0 log<sub>2</sub> or higher, there was complete protection against a high challenge dose of Asian H5N1 HPAI (Ellis et al., 2004). A close relationship between HI titers generated by plasmid DNA (greater than 1:40) and protection was observed previously in mice, ferrets and non-human primates immunized with a plasmid encoding consensus HA gene of H5 avian influenza virus (Laddy et al., 2008). In the present studies a pilot trial



was conducted (data not shown) in which 8-week-old unvaccinated SPF chickens were subjected to a challenge with a dose of 0.2 mL ( $10^{6.17}$  EID<sub>50</sub>/0.1 mL) of A/duck/Anhui/269/2002 (H6N2) by intranasal instillation. There were no measurable clinical effects so it was decided not to include virus challenge studies of the H6 DNA vaccinated chickens and we can make no claims about the protective efficacy of these constructs. Nevertheless, one can expect that if protection against avian influenza infection is equated with a HI titer of 5.0 log<sub>2</sub> or higher, seven out of eight chickens in the 100 µg EP group and two (pCAG-HAk) or three (pCAG-optiHAk) out of eight birds in the 100 µg IM group had achieved this level of protection.

EP has been well documented to increase the magnitude of the humoral and cellular responses induced by DNA vaccines in a range of animal models such as mice (Muthumani et al., 2008; Widera et al., 2000), rats (Zucchelli et al., 2000), rabbits (Wang et al., 2008; Widera et al., 2000; Zucchelli et al., 2000), guinea pigs (Widera et al., 2000), goats and cattle (Tollefsen et al., 2003), sheep (Babiuk et al., 2007) and non-human primates (Luckay et al., 2007). In this study, all chickens vaccinated with the pCAG-HAk DNA vaccine via EP generated high titers of H6 antibody, comparable to titers previously achieved with an inactivated whole virus H6N2 vaccine in other experiments (Shan, 2010). The mean H6 HI titer was  $\geq 8$ -fold higher in the EP vaccinated group than in the IM vaccinated group from 2 to 8 weeks post-vaccination. This indicated that vaccination with EP was significantly more effective than routine IM inoculation with the pCAG-HAk vaccine in chickens.

In the current study, the virus HA gene sequence was also replaced by the chicken biased codon sequence, resulting in 25.2% alteration at the nucleotide level and 18.2% GC ratio

increase in the codon-optimized HA DNA sequence, compared to the wild type virus sequence. The high GC content in the gene may be favorable for its mRNA stability, processing and nucleo-cytoplasmic transport (Haas et al., 1996; Jiang et al., 2007). This high GC rate could increase the number of CpG motifs in an optimized gene, which may act as an inherent adjuvant. Based on codon alteration, improvements in the immunogenicity of the optimized HA-encoding plasmid were expected, however, the codon optimized HA DNA vaccine did not achieve better immunogenicity in terms of seroconversion rate and HI titer than the wild type HA-expressing DNA vaccine. This has similarities with other studies in which re-engineering the coding sequence with the most prevalent HIV-1 codons impaired considerably rat thymocyte protein (Thy-1) expression, but enhanced green fluorescent protein expression (Haas et al., 1996).

Many factors regulate and influence gene expression levels. It has been assumed widely that translational initiation is responsible for translational efficiencies of mammalian gene products (Haas et al., 1996). The toeprinting assay used with mRNAs showed that the nucleotide G in position +4 augmented recognition of AUG and favored translation (Kozak 1987). Using the same codon optimization strategy as the current study for an optimized HA gene from A/goose/Guangdong/1/96 (H5N1) the first amino acid following ATG was Glu (GAG) and this construct achieved the expected increased immune response (Jiang et al., 2007). In contrast, in the current study with the optimized H6-HA gene, the first amino acid after ATG was Ile (ATC) and the nucleotide at the +4 position was A rather than G. This may have accounted for some suboptimal expression in chickens. Additionally, some factors such as mRNA secondary structure, internal TATA boxes, cryptic splicing sites, premature Polka sites, internal chi sites, ribosomal

binding sites, negative CpG islands, RNA instability motif (ARE) and inhibition sites may all impact on ribosomal binding and stability of mRNA (Wang et al., 2006). Thus, expression of a codon biased gene may be further enhanced by RNA optimization. The codon optimization approach in this study needs to be further explored.

The promoter driving gene expression was one of the factors affecting the efficacy of DNA vaccines (Suarez and Schultz-Cherry 2000). Previous experiments showed that four expression vectors (VR1012, pCI, pCI-neo and pVAX1) expressing the same HA gene under the control of the cytomegalovirus (CMV) promoter induced no or minimal antibody response in Hy-line chickens (Shan, 2010), even when co-administered with lipofectin as adjuvant. In the current study, insertion of the Kozak sequence and use of the pCAGGS vector, which contains an intron between the promoter and the HA ORF, under the control of the chicken  $\beta$ -actin promoter contributed to a higher level of H6 HA expression than the four DNA vaccines in previous studies, resulting in a detectable antibody response. Analyzing the parameters which impact on DNA-induced antibody responses is important to successfully develop DNA vaccines. Further investigation of these factors with this DNA construct is currently being undertaken.

To conclude, this study showed that DNA vaccines can induce antibody response in chickens to H6N2 avian influenza virus by selection of an appropriate vector and insertion of a Kozak sequence. Electroporation was an effective method to enhance the potency of DNA vaccines. However, codon optimization did not seem to demonstrate the expected enhancing effect.

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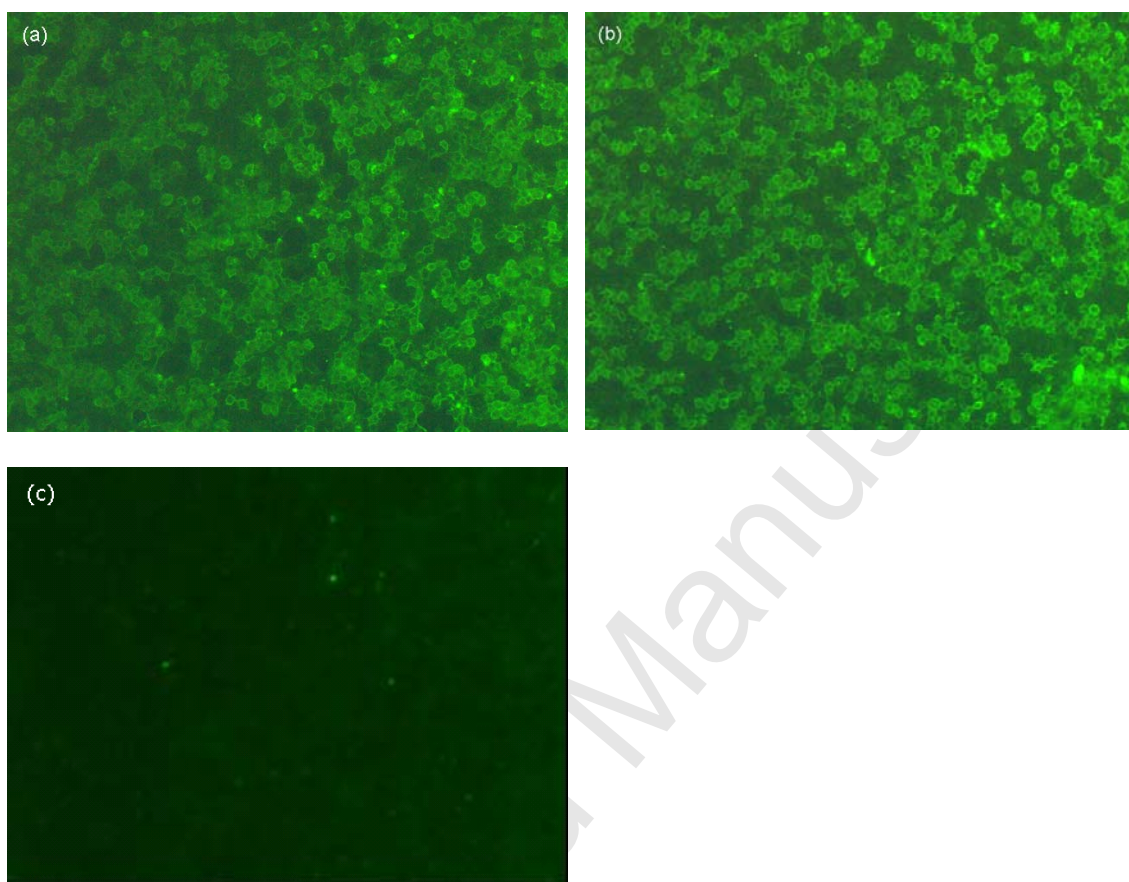
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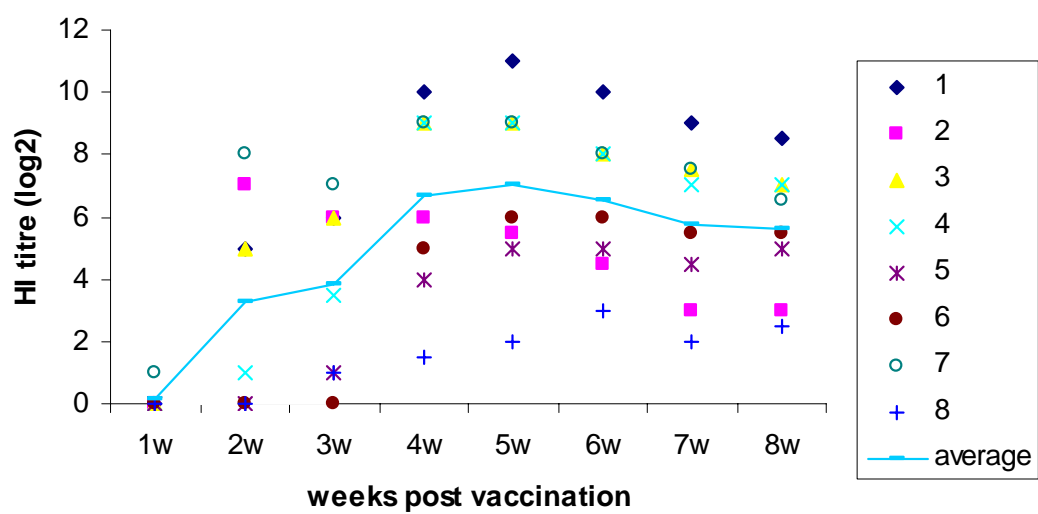
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Fig. 1. Photomicrographs of immunofluorescent staining for H6 avian influenza antigens in 293T cells transfected with (a) pCAG-optiHAk, (b) pCAG-HAk, (c) pCAGGS only. (Leica DMIRES2, x100).

Fig. 2. Temporal changes in H6 HI titer over an 8-week period post-vaccination in chickens immunized with 100  $\mu$ g pCAG-HAk via EP. The curve represents the mean antibody titer of the group. Each icon number refers to one bird.

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