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Fusion of HSP70 gene of *Mycobacterium tuberculosis* to hemagglutinin (H5) gene of avian influenza virus in DNA vaccine enhances its potency

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Summary. – A series of plasmids containing the HSP70 gene of *Mycobacterium tuberculosis* fused to the hemagglutinin (H5) gene of H5N1 avian influenza virus (AIV) (H5-HSP70 (heat shock protein 70) vaccine) or individual H5 gene (H5 vaccine) or HSP70 gene (HSP70 vaccine) were constructed based on the plasmid pcDNA3.1. Expression of H5 gene in Vero cells *in vitro* and in chickens *in vivo* was confirmed following their transfection and immunization with H5 or H5-HSP70 vaccines. Controls consisted of HSP70 vaccine, empty plasmid pcDNA3.1 and co-administered H5 and HSP70 vaccines. H5-HSP70 vaccine produced in chicken higher hemagglutination inhibition (HI) antibody titer than H5 vaccine. However, the increase was not statistically significant. We have demonstrated for the first time that the H5 DNA vaccine with fused HSP70 gene may produce an enhanced induction of humoral immune response to AIV in chickens.

Keywords: avian influenza virus; hemagglutinin H5; HSP70; DNA vaccine; humoral immunity

Introduction

Influenza A viruses (the family *Orthomyxoviridae*) are important veterinary and human pathogens. The influenza virus genome is segmented and contains 8 single-stranded, negative sense RNA strands that code for 11 proteins (De Jong and Hien, 2005). Two types of surface glycoproteins e.g. hemagglutinin (HA) and neuraminidase (NA) are protruding from the virion.

Vaccination against AIV is an important option, especially when large populations of susceptible poultry are close to the infected area (Normile, 2004). Currently used vaccines contain complete inactivated AIV (Villarreal-Chavez, 2007). However, new technologies have been implemented to produce the advanced vaccines, e.g. reverse genetic-engineered AIV vaccine, baculovirus-derived H5 and H7 subunit vaccines (Johansson, 1999, Hoffmann *et al.*, 2000) and fowl poxvirus recombinant vaccine containing H5 or H7 genes (Boyle *et al.*, 2000). Although these vaccines induce a protection against AIV, they have many limitations including minimal cross-protection to the diverse influenza strains (Swayne *et al.*, 2001). The viruses that cause greatest difficulties in vaccine production are highly pathogenic avian influenza viruses (HPAIVs) H5, H7, and H9 subtypes. These viruses require a biosafety facility of high level for handling. In some cases, these viruses are not able to give a high yield of virus in the embryonated chicken eggs, because HPAIVs are lethal to the chicken embryo and kill the embryo before a virus can be harvested (Wood, 2001). H5N1 DNA vaccines are safe and effective in experimental animals (Liu *et al.*, 2003). There are numerous studies describing DNA vaccination of different animals indicating the ability of DNA vaccines encoding HA, NA, M1, and NP proteins of AIV to induce specific humoral

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Abbreviations: AIV = avian influenza virus; HA= hemagglutinin; HI = hemagglutination inhibition; HIV-1 = Human immunodeficiency virus; HPAIV(s)= highly pathogenic avian influenza virus(es); HSP(s)= heat shock protein(s); LPAIV= low pathogenic avian influenza virus; NA = neuraminidase

immune response (Kodihalli *et al.*, 1997; Lunn *et al.*, 1999). Hence, it may be possible to improve the next generations of DNA vaccines by incorporating additional genes against multiple subtypes (H5, H7, H9) into a plasmid and/or use more conserved genes like NP and M, which can induce cross-protection against different AIV subtypes.

HSPs are highly conserved intracellular molecules that are found in eukaryotic and prokaryotic species. They are responsible for maintaining cell homeostasis participating in a number of protein house-keeping functions including a protein folding, prevention of protein aggregation and misfolding (Jaattela, 1999). HSP-based vaccination has produced promising results for therapeutic and prophylactic purposes (Srivatava and Malki, 1991; Noessner *et al.*, 2002). HSP70 prepared from cancer cells or virus-infected cells is capable of eliciting $CD8^+$ CTL response *in vivo* and *in vitro* (Srivastava, 2002). HSP70 molecules act as an antigen carrier, since they contain T-cell epitopes resulting in production of antigen-specific B cells and $CD4^+/CD8^+$ T cell response (Udono *et al.*, 2001). The adjuvant effect of HSPs is commonly studied by fusion of HSP with an antigen of interest. Fusion of HSP70 to nucleoprotein of Hantaan virus significantly increased the level of nucleoprotein-specific antibody in mice (Li *et al.*, 2007). Vaccination with fusion protein containing EB200 protein of *Plasmodium falciparum* fused to the C-terminal part of HSP70 could significantly increase Th-1 antibody response and interferon γ production (Qazi *et al.*, 2005). Meanwhile, fusion of Human immunodeficiency virus 1 (HIV-1) p24 to the N-terminus of mycobacterial HSP70 can elicit both humoral and cellular immune response against p24 in mice (Suzue and Young, 1996).

The aim of this study was to test a potential enhancement of immunogenicity of an AIV DNA vaccine, in which H5 gene was fused with HSP70 gene. For this purpose, a series of DNA vaccine plasmids containing the fused H5-HSP70 gene or individual H5 or HSP70 gene were constructed and tested for the expression in Vero cells *in vitro* and for the immunogenicity in chickens *in vivo.*

Materials and Methods

Vaccine plasmid constructs. To construct the eukaryotic expression plasmid DNA vector for H5, the full length H5 DNA was amplified from pCR2.1/H5 vector (Invitrogen) by PCR using primers (forward: 5'-CCCC**AAGCTT**ATGGAGAAAATAGTGCTT-3'; reverse: 5'-CCC**GGATCC**AATGCAAATTCTGCATTGTAA-3') that introduced to the amplicons *Hind*III and *Bam*HI sites (boldface), respectively. The H5 gene was originally isolated from the H5N1 virus (A/chicken/H5N1/5858/2004) isolated from Pasir Pekan, Kelantan, Malaysia. To construct eukaryotic expression plasmid pcDNA3.1(+) with *M. tuberculosis* HSP70 gene, the complete ORF of HSP70 was amplified from plasmid pMRLB6 that was kindly obtained from the Department of Microbiology, Immunology, and Pathology (Colorado State University, USA). The primers (forward: 5'-AAAA**GGATCC**ATGGCTCGTGCGGTCGGGATCG-3'; reverse: 5'-AAAA**CTCGAG**TCACTTGGCCTCCCGGCCGTC-3') introduced to the amplicons *Bam*HI and *Xho*I sites (bold face), respectively. The PCR amplified H5 and HSP70 DNA were digested with *Hind*III/*Bam*HI and *Bam*HI/*Xho*I (Fermentas), respectively, and ligated with T4 ligase (Vivantis) to pcDNA3.1 (+) expression vector (Invitrogen) downstream of HCMV promoter and termed as H5 vaccine and HSP70 vaccine. To construct a DNA vector containing the fused form of H5-HSP70, the HSP70 was amplified by PCR using the primers mentioned above and sub-cloned to the 5' flank of the H5 plasmid at the *Bam*HI and *Xho*I sites resulting in H5-HSP70 vaccine. After ligation, the reconstructed plasmids were transformed into competent *Escherichia coli* Top 10 and cultured overnight.

PCR for H5, HSP70, and H5-HSP70 genes. The presence of the insert was identified by the colony PCR screening and restriction enzyme digestion, which were carried out using the primers and PCR conditions that was used to amplify the different genes. All constructs were further verified by DNA sequencing (Applied Biosystems). For transfection and immunization, the plasmids were prepared from overnight *E. coli* TOP 10 and purified using the EndoFree plasmid Mega purification system (Qiagen) according to the manufacturer's instructions. DNA concentration and purity was determined by the measurement of A_{260} and A_{280}.

Transfection of Vero cells. Prior to DNA immunization, the expression efficiency of eukaryotic expression vector was assessed *in vitro* by transient transfection of Vero cells. The cells were cultured in RPMI medium with L-glutamine and 25 mmol/l HEPES buffer (GIBCO) supplemented with 10% FBS (Hyclone) and 1% Penicillin/Streptomycin (GIBCO). Transient transfection was performed by mixing 2 μg of plasmid DNA with FuGENE® HD Transfection Reagent (Roche). The transfection complex was added to the cells in drop-wise manner and incubated overnight at 37°C in a CO_2 incubator. The cells were harvested at 72 hrs post-transfection and the protein expression was detected by Western blot analysis.

Western blot analysis. The cell lysates were run on SDS-PAGE and electroblotted to nitrocellulose membrane. The blots were incubated with monoclonal antibody against HSP70 diluted at 1:1000 (Colorado State University, USA) or rabbit polyclonal antibody against AIV hemagglutinin A/chicken/Jilin/9/2004 (H5N1) diluted at 1:2,000 (Abcam). The detection of HSP70 and H5 proteins was performed by using of anti-mouse and anti-rabbit secondary antibody, respectively, diluted at 1:5,000 (Invitrogen). The blots were incubated with chromogenic substrate (BCIP/NBT substrate for alkaline phosphatase) until purple bands developed.

DNA vaccination of chickens. SPF eggs were purchased from Malaysian Vaccine Pharmaceutical. Hatched chickens were randomly divided to 6 groups. Four groups were inoculated with H5, H5-HSP70, HSP70 vaccines and pcDNA3.1, respectively. The 5th group received co-administration of H5 and HSP70 vaccines, while the 6th group was left as a control. Prior to the immunization, blood samples were taken from all the chickens and used as pre-immune control sera. Ten-day-old chicks were injected 3 times with 100 μg of the respective plasmid DNAs at 2-week interval. Tuberculin syringes (1 ml) with attached 27 G x ¾ inch (0.4 mm x 20mm) needle were used to inject 100 μg of DNA solutions intramuscularly into pectoral muscle. Blood samples were collected weekly at days 7, 17, 24, 31,

38, and 45 of immunization. The serum was tested for the presence of H5 antibodies using ELISA and HI test. A group of chickens was kept as a positive control after single immunization with inactivated $10^{6.3}$ EID_{50} of inactivated A/Duck/Malaysia/ 8443/2004 (H5N2) administered subcutaneously. Immunized 52-day-old chickens were euthanized and tissue samples such as pectoral muscle and spleen were collected and stored at -20°C until used.

ELISA. For primary screening of the H5-specific antibodies in the sera of immunized chickens the ELISA kit AniGen H5 AIV Ab (Anigen Animal Genetic) was employed. This kit has been recommended by several reference laboratories for qualitative detection of antibodies to the most common and prevalent AIV in chickens and ducks. The A_{405} value with the reference A_{620} was taken. The percent inhibition (PI) value for each serum sample was calculated using formula: PI = [1 – (A_{405} sample/mean A_{405} negative)] x 100. PI value equal or greater than 50 was considered as positive for AIV.

Hemagglutination inhibition test. HI test was performed in a 96-well microtiter plate with 0.6% chicken red blood cells as described by Kendal *et al.* (1982) using an inactivated AIV, strain A/My/H5N2/Duck/2004 kindly supplied by Dr. M. Mohamed from Veterinary Research Institute, Ipoh, Malaysia. Sera from chickens were tested individually after treatment with incubation at 58°C for 30 mins. Chicken sera were tested individually and HI antibody titers were determined as the reciprocal of the highest serum dilution that gave complete HI.

RT- PCR for H5 transcripts. Tissue samples were homogenized using a hand-held pellet pestle and RNA was extracted as per the manufacturer's direction using guanidine isothiocyanate based reagent (TRI Reagent® Life Technologies). The concentration and purity of extracted RNA was determined by spectrophotometer (BioRad Smartspec ™ 3000) according to the method described by Wilkinson (1993). One-step RT-PCR was performed using one-step Access RT-PCR system (Promega) following the manufacturer's protocol with slight modification. The RT-PCR amplification was performed in final volume of 25 µl containing AMVITfl 5X, reaction buffer 1X, MgSO4 (3 mmol/l), dNTP Mix (0.2 mmol/l), forward primer 5'-TCCAAAGTAAACGGGCAAAG-3' (0.2 µmol/l), reverse primer 5'-TGYTG AGTCCCCTTTCTTGA-3' (0.2 µmol/l), RNasin® Ribonuclease inhibitor (0.8 U/µl), AMV reverse transcriptase (0.1 U/µl), Tfl DNA polymerase (0.1 U/µl), RNA template (~ 100 ng/µl) and water. The amplification was performed in thin walled tube using Mycycler Gradient (BioRad) with heated lid. The amplified RT-PCR products were electrophoresed using 2% agarose gel.

Statistical analysis. HI antibody titer obtained from individual chicken was analyzed using SAS, version 9.1. The unpaired Student's *t*-test was performed for statistical analysis and data were presented as mean ± SD. Values of $P \leq 0.05$ were considered statistically significant.

Results

Construction of vaccine plasmids and expression of H5, HSP7, and H5-HSP70 proteins in vitro

PCR screening analysis identified a single band of expected size of 1.7 and 1.9 kb for H5 and HSP70 constructs, respectively. In the case of H5-HSP70 construct, single band of 3.6 kb was observed (Fig. 1). The nucleotide sequences introduced into vectors were further confirmed by an automated sequencing and matched H5 and HSP70 sequences deposited in the GenBank. Afterwards, the cloned HSP70 gene was ligated in-frame with H5 plasmid. Sequence analysis also revealed the expected restriction enzyme sites, *Bam*HI and *Xho*I at the 5'- and 3'-end of the inserts, respectively.

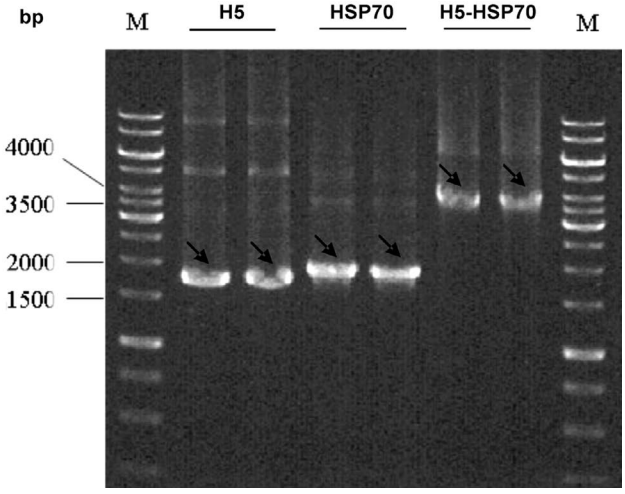

Fig. 1

Identification of vaccine plasmid constructs

Agarose electrophoresis of PCR products. The arrows indicate H5, HSP70, H5-HSP70. DNA size markers (lanes M).

The ability of constructed recombinant plasmid H5, HSP70, and H5-HSP70 to express the proteins of interest was determined in Vero cells transfected with the respective plasmids. Western blot analysis detected the bands of expected size of approximately ~70 K for HSP70 protein and ~69 K for H5 protein in the cells transfected with HSP70 and H5 constructs, respectively (Fig. 2a,b). Whilst, a large stained band of the expected size of ~140 K was observed in the cells transfected with H5-HSP70 construct (Fig. 2c).

Detection of H5 transcripts in tissues of DNA-vaccinated chickens

The ability of the constructed plasmids, H5 and H5-HSP70, to induce mRNA expression of H5 and H5-HSP70 in chickens was examined by RT-PCR. The presence of H5 transcripts in the muscle and spleen of chickens inoculated with H5 and H5-HSP vaccines was detected as the bands of the expected size between 100 to 200 bp (Fig. 3). Based on the nucleotide sequence of H5 primers, the exact length of the PCR product is 140 bp. In order to rule out the possibility that amplification from RT-PCR is not due to the plasmid DNA, the samples were subjected to PCR amplification without the

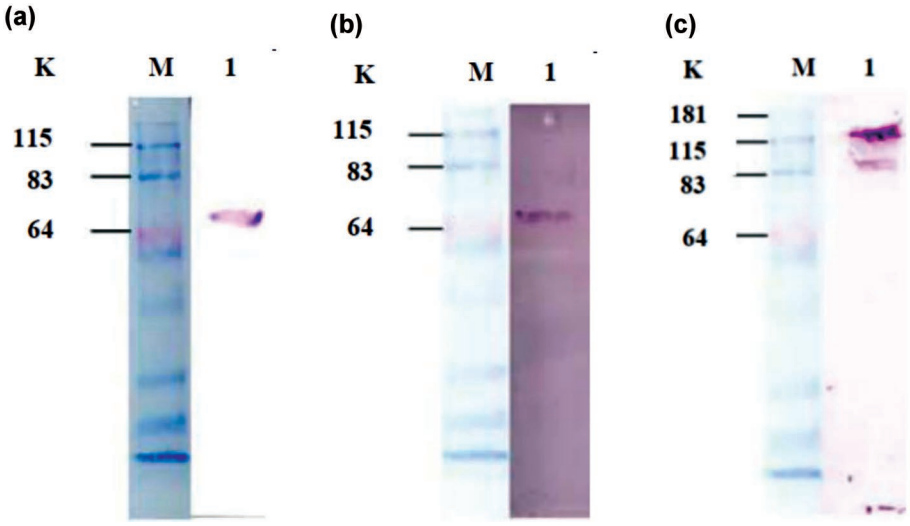

Fig. 2

Expression of vaccine plasmid constructs in Vero cells

Western blot analysis. HSP70 vaccine (a), H5 vaccine (b), H5-HSP70 vaccine (c). Protein size markers (lanes M).

RT step. No band of the expected size was detected indicating that the amplified band was associated with H5 transcription from the inoculated DNA vaccines (data not shown).

Antibody response in DNA vaccinated chickens

To investigate the capacity of the recombinant DNA vectors to elicit influenza H5-specific antibody response, chickens were immunized with H5, H5-HSP70, HSP70, or co-administered with H5 and HSP70 vaccines. The level of H5-specific antibodies in the sera of immunized chickens was analyzed by ELISA and HI test. Only chickens immunized with H5 and H5-HSP70 vaccines developed antibodies against AIV (Table 1). The control chickens inoculated with pcDNA3.1 or HSP70 did not show any presence of AIV antibodies. The antibody titers remained undetectable 2 weeks after the first administration in most of the immunized chickens. However, the antibodies against AIV were

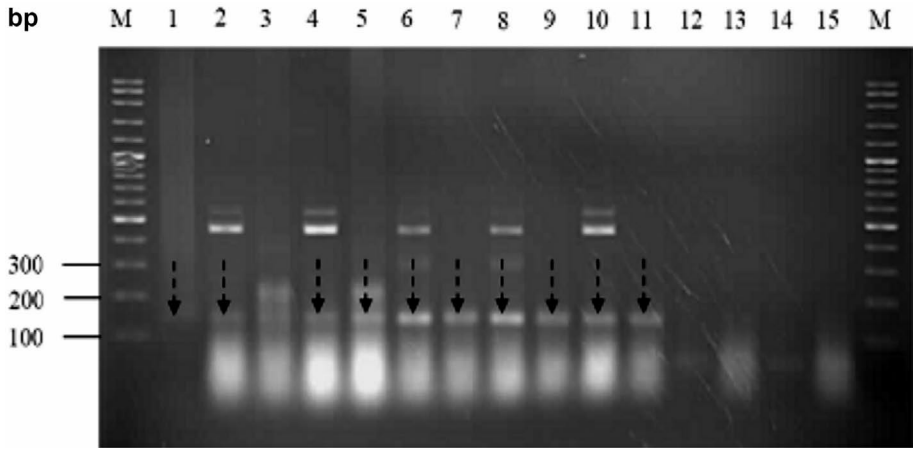

Fig. 3

Detection of H5 transcripts in tissues of DNA-vaccinated chickens

Agarose electrophoresis of RT-PCR products. DNA size marker (lanes M), positive control (lane 1), H5 vaccine (lanes 2, 3), co-administered H5 and HSP70 vaccines (lanes 4–7), H5-HSP70 vaccine (lanes 8–11), HSP70 vaccine (lanes 12, 13), negative controls (lanes 14, 15). The arrows indicate H5 transcript (140 bp).

detected in majority of the chickens immunized with H5-HSP70 vaccine at day 21 post immunization. The number of chickens positive for H5 antibody increased in 4th and 5th week post vaccination. We found 5 positive chickens in the group vaccinated with H5-HSP70 (Table 1).

HI test was performed using H5 antigen derived from a low pathogenic AIV (LPAIV), H5N2. Sequence analysis of H5 gene from H5N1 and H5N2 that was used in this study showed nucleotide and amino acid identity of more than 87% (data not shown). Serum samples of the vaccinated birds had zero or very low HI antibody titers in the blood samples taken on day 14 and 21 post vaccination (Table 2).

Chickens in the groups immunized with H5 and H5-HSP70 vaccines began to develop HI antibody titer as early as 3 weeks after the first immunization (Table 2). However, the antibody titers remained low with an average HI antibody titer of 2.3 ± 0.8 and 3.0 ± 1.1 in the groups immunized with H5 and H5-HSP70 vaccines , respectively. Four weeks after the immunization, HI antibody titer increased further to 5.0 ± 2.4 and 7.3 ± 4.7 in groups immunized with H5 and H5-HSP70, respectively. At five weeks post immunization, HI antibody titers increase to an average of 10.8 ± 4.1 and 17.3 ± 11.8 in both groups immunized with H5 and H5-HSP70, respectively. Serum of the chickens immunized with inactivated H5N2 served as the positive control and showed high level of HI antibody titers of 14.1 ± 4.5 at day 21 post immunization. Hence, compared to the chickens immunized with H5, a higher individual antibody titer was observed in chickens immunized with H5-HSP70 vaccine at day 21, 28, and 35 post immunizations (Table 2). However, the increase in the antibody titers was not significantly different ($P \leq 0.05$). The control chickens inoculated with HSP70 and pcDNA3.1 vector showed no antibody response detectable by ELISA or HI test.

Table 1. H5 antibodies in sera of DNA-vaccinated chickens determined by ELISA

DNA vaccine	Days post vaccination				
	7	14	21	28	35
	Positive/total				
H5	0/6	0/6	2/6	3/6	4/6
H5-HSP70	0/6	0/6	2/6	4/6	5/6
H5 + HSP70	0/6	0/6	2/6	3/6	4/6
HSP70	0/6	0/6	0/6	0/6	0/6
pcDNA3.1	0/6	0/6	0/6	0/6	0/6

Discussion

Several different types of vaccines against H5N1 are available for the commercial use. Most of them are based on inactivated LPAIV of H5 subtypes i.e. H5N2 and H5N9 (Villarreal-Chavez, 2007) and live recombinant fowlpox virus expressing H5 of LPAIV (Qiao *et al.*, 2003). This study describes the use of HSP70 to enhance an efficiency of DNA vaccine against AIV in chickens. Immunization of chickens with H5 and H5-HSP70 vaccines induced detectable antibodies to H5 confirmed by ELISA and HI test. This study demonstrated that immunization of chickens with HSP70 based H5 DNA vaccine could produce higher antibody titer compared to the groups immunized with H5 alone or co-administered with HSP70. Three weeks after the first immunization, all chickens immunized with H5, H5-HSP70, and H5 + HSP70 vaccines produced detectable antibody response. The highest antibody response was observed in chickens vaccinated with H5-HSP70 vaccine after the third vaccination reaching HI antibody titer of 17.3 in average. ELISA results indicated that 5 out of 6 chickens immunized with H5-HSP70 were positive. However, the increase in comparison with chickens immunized with H5 vaccine alone was not statistically significant ($P \leq 0.05$), what could be due to a high standard deviation and to the limited number of chickens. Moreover, chickens vaccinated with equal amounts of either empty plasmid pcDNA3.1 or HSP70 alone did not produce any antibody response against HA.

According to the manufacturer, the commercial ELISA used in this study had 94% relative sensitivity and 100% relative specificity. It was shown that HI test is more sensitive than ELISA in detecting AIV specific antibody (Zarkov,

Table 2. HI antibody titers in sera of DNA-vaccinated chicken

DNA vaccine	Days post vaccination				
	7	14	21	28	35
	HI antibody titers ± SD				
H5	ND	0.7 ± 1.0	2.3 ± 0.8	5.0 ± 2.4	10.8 ± 4.1
H5-HSP70	ND	ND	3.0 ± 1.1	7.3 ± 4.7	17.3 ± 11.8
H5 + HSP70	ND	ND	1.8 ± 0.4	5.0 ± 2.5	10.8 ± 4.1
HSP70	ND	ND	ND	ND	ND
pcDNA3.1	ND	ND	ND	ND	ND
Control antigen-inactivated H5N2	ND	ND	7.2 ± 3.2	9.7 ± 4.4	14.1 ± 4.5

ND = not detected.

2006), what can explain the difference between ELISA and HI titers. Furthermore, the variation in antibody titers was probably associated with the differences in assay formats and antigenicity of the H5N2 virus used. However, many studies on the efficacy of HSP fusion protein vaccines are inconsistent. Vaccination with Human papillomavirus HPV-16 protein E7 fused to HSP70 as a DNA vaccine did not generate E7-specific antibodies. However, E7-HSP70 fusion genes increased the frequency of E7 specific CD8+ T cells by 30-folds relative to the vaccine containing a wild type E7 gene (Chen *et al.*, 2000). Further, studies on Hantaan virus, HIV-1, *P. falciparum* and *P. malariae* have demonstrated the adjuvant properties of HSP70 (Suzue and Young 1996; Qazi *et al.*, 2005; Li *et al.*, 2007). In these studies, the antigen was positioned either at N- or C-terminus of the HSP and the size of the antigen fusion partner varied from 142 to 433 aa. In our study, H5 was fused to the N-terminus of mycobacterial HSP70 and produced higher HI antibody titer, but this increase was not significant. The size of mycobacterial HSP70 used in our study was bigger than 550 aa.

Some previous studies reported that HA DNA vaccine failed to induce detectable pre-challenge HI antibody in non-human primates, mice, ferrets, and pigs (Liu *et al.*, 1997; Macklin *et al.*, 1998; Kodihalli *et al.*, 1999). Nevertheless, direct intramuscular injection, at least in chickens, produced variable immune responses (Liu *et al.*, 1997). However, in our study, direct intramuscular injection of H5 and H5-HSP70 vaccines produced detectable levels of HI antibodies, what was consistent with the previous results (Lee *et al.*, 2006). Other studies also showed that a proper selection of promoter/enhancer element to drive the expression of transferred genes was critical for the successful gene delivery. This choice depended upon both, the target cell type and the functional design of the vector construct (Kodihalli *et al.*, 1997). It has also been shown that the antibody response in chickens induced by the plasmids can vary in response due to the differences in codon biases for protein translation in different species, what can contribute for partial protection or variations of antibody response in chickens (Jiang *et al.*, 2007). Hence, the use of chicken-optimized codons in HA gene may allow for a great increase in the expression of this gene in chickens. The low HI titer detected in the vaccinated birds might be also due to the highly immunogenic nature of the mycobacterial HSP70 protein, which might have directed the immune response to produce HSP70 antibodies. However, in this study the antibody response against HSP70 was not evaluated.

The detection of H5 transcripts in spleen of immunized mice indicated that DNA was delivered to the immune system. In the same way, it indicated the capacity of DNA vaccines to deliver targeted genes to the various tissues. Many studies showed that DNA vaccines was detectable in the serum and different tissues including the site of injection, heart, liver, lung, spleen and bursa even 25 days post immunization (Winegar *et al.*, 1996; Oh *et al.*, 2001, You *et al.*, 2007). The results of our study demonstrated that H5 and H5-HSP70 DNA vaccines had the potential to be used as vaccines against AIV. However, further studies have to be conducted concerning cellular immune response in particular. Even though low HI antibodies against H5 influenza virus were detected after two DNA injections, 100% of the DNA-vaccinated chickens were protected against homologous challenge and 95% against two H5 variants that otherwise cause lethal infection in chicken (Kodihalli *et al.*, 1997).

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