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Vaccination with recombinant 4 × M2e.HSP70c fusion protein as a universal vaccine candidate enhances both humoral and cell-mediated immune responses and decreases viral shedding against experimental challenge of H9N2 influenza in chickens

Mehran Dabaghian [a,b], Ali Mohammad Latify [a], Majid Tebianian [c], Hassan Nili [d], Ali Reza Tevangar Ranjbar [e], Ali Mirjalili [c], Mashallah Mohammadi [c], Reza Banihashemi [f], Seyyed Mahmoud Ebrahimi [a,*]

[a] Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, PO Box 14155-3651, Tehran, Iran
[b] Department of Pathobiology, University of Tehran, Faculty of Veterinary Medicine, PO Box 14155-6453, Tehran, Iran
[c] Department of Biotechnology, Razi Vaccine and Serum Research Institute (RVSRI), PO Box 31975/148, Karaj, Tehran, Iran
[d] Department of Avian Research, School of Veterinary Medicine, Shiraz University, PO Box 1731, Shiraz, Iran
[e] Department of Medical Virology, Iran University of Medical Sciences, Tehran, Iran
[f] Department of Medical Immunology, Tarbiyat Modares University, Tehran, Iran

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ABSTRACT

As cellular immunity is essential for virus clearance, it is commonly accepted that no adequate cellular immunity is achieved by all available inactivated HA-based influenza vaccines.

Thus, an improved influenza vaccine to induce both humoral and cell-mediated immune responses is urgently required to control LPAI H9N2 outbreaks in poultry farms.

M2e-based vaccines have been suggested and developed as a new generation of universal vaccine candidate against influenza A infection.

Our previous study have shown that a prime-boost administration of recombinant 4 × M2e.HSP70c (r4M2e/H70c) fusion protein compared to conventional HA-based influenza vaccines provided full protection against lethal dose of influenza A viruses in mice. In the present study, the immunogenicity and protective efficacy of (r4M2e/H70c) was examined in chickens.

The data reported herein show that protection against H9N2 viral challenge was significantly increased in chickens by injection of r4M2e/H70c compared with injection of conventional HA-based influenza vaccine adjuvanted with MF59 or recombinant 4 × M2e (r4M2e) without HSP70c. Oropharyngeal and cloacal shedding of the virus was detected in all of the r4M2e/H70c vaccinated birds at 2 days after challenge, but the titer was low and decreased rapidly to reach undetectable levels at 7 days after challenge. Moreover, comparison of protective efficacy against LPAI H9N2 in birds intramuscularly immunized with r4M2e/H70c likely represented the ability of the M2e-based vaccine in providing cross-protection against heterosubtypic H9N2 challenge and also allowed the host immune system to induce HA-homosubtype neutralizing antibody against H9N2 challenge.

* Corresponding author. Tel.: +98 261 4570038 46; fax: +98 261 4552194.
E-mail addresses: sme3422@gmail.com, smebrahimi@shirazu.ac.ir (S.M. Ebrahimi).

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This protective immunity might be attributed to enhanced cell-mediated immunity, which is interpreted as increased lymphocytes proliferation, increased levels of Th1-type (IFN-γ) and Th2-type (IL-4) cytokines production and increased $CD4^+$ to $CD8^+$ ratios, resulting from the injection of four tandem repeats of the ectodomain of the conserved influenza matrix protein M2 (4 × M2e) genetically fused to C-terminus of *Mycobacterium tuberculosis* HSP70 (mHSP70c).

1. Introduction

Avian influenza viruses (AIVs) pose a threat toward the health of both poultry and human population worldwide. However, in many countries, low pathogenic avian influenza H9N2 (LPAI H9N2) viruses are an enormous economic burden on the commercial poultry industry when they cause signs of mild respiratory disease and a reduction in egg production (Alexander, 2003; Senne, 2003). Some reports also revealed that the LPAI H9N2 viruses in domestic poultry farms resulted in sever clinical signs and high mortality depending on the viral strain as well as co-infection with other pathogens or immunosuppressive diseases (Bano et al., 2003; Kishida et al., 2004).

Vaccination using conventional HA-based vaccine is the major tool for the prevention and control of LPAI H9N2 viruses in the poultry industry where the HA of the vaccine strain matches that circulating virus (Boni, 2008; Sun et al., 2012).

Conventional HA-based influenza vaccine has been proved to elicit strong humoral responses and can prevent clinical disease and reduce viral shedding in field conditions, but it cannot prevent vaccinated poultry from becoming infected and from shedding wild viruses in farm settings (Swayne and Halvorson, 2003; Sun et al., 2012).

Moreover, in contrast to hemagglutinin (HA) protein, the matrix protein 2 (M2e) of influenza virus is not accessible to antibody and therefore antibodies to M2e are not virus-neutralizing. However, it was reported that anti-M2e antibodies can bind to the M2e protein on infected host cells and reduce virus replication by interfering with virus budding and by mediating the killing of infected cells by complement or by cells of the innate immune system (Jegerlehner et al., 2004; Subbarao and Joseph, 2007; Mozdzanowska et al., 1999).

As cellular immunity is essential for virus clearance, it is commonly accepted that no adequate cellular immunity is achieved by currently available influenza vaccine (Choi et al., 2008; Wareing and Tannock, 2001).

Thus, an improved influenza vaccine to induce both humoral and cell-mediated immune responses is urgently required to control LPAI H9N2 outbreaks in poultry farms.

Additionally, conventional HA-based vaccines are mainly designed to induce subtype-specific neutralizing antibodies and do not protect against infection with other influenza subtypes or with antigenic variants (Fan et al., 2007; Brett and Johansson, 2005). Hence, new vaccination strategies that will result in broad cross-reactivity against influenza viruses need to be developed.

So far, several approaches have been investigated to develop broadly protective vaccines and focus mainly on the conserved region of the viral matrix protein-2 (M2) protein of influenza A virus. M2e-based vaccines have been suggested and developed as a new generation of universal vaccine candidate against influenza A infection.

Indeed, M2e protein with different constructions, including ones from our laboratory, revealed a high potential as a vaccine for prevention of highly pathogenic (homologous and heterologous) influenza A viruses in animal models (Misplon et al., 2010; Fan et al., 2004; Slepushkin et al., 1995; Frace et al., 1999; Neirynck et al., 1999; Mozdzanowska et al., 2003; Huleatt et al., 2008; Song et al., 2011; Ebrahimi et al., 2012; Zabeh Jazi et al., 2012). Previous studies have reported on antibodies and cell-mediated cytotoxicity specific to the M2e antigen and their anti-viral activity (Jegerlehner et al., 2004). It was shown previously that nucleotide based CpG oligodeoxynucleotides (CpG-ODN) adjuvant with M2e peptide and *Escherichia coli* expressed M2e protein fused with activation associated protein-1 (ASP-1) or Salmonella typhimurium flagellin proteins 2 (STF2) adjuvant significantly induced both humoral and cell-mediated immune responses in mice with M2e-specific IgG2a and IFN-γ secreting lymphocytes (Wu et al., 2009; Zhao et al., 2010; Huleatt et al., 2008).

In our previous study, it was shown that a prime-boost administration of 4 × M2e.HSP70c without adjuvant by intramuscular route in mice (Balb/c) provided full protection against lethal dose of mouse-adapted H1N1, H3N2, or H9N2 influenza A isolates circulating in Iran. However, protection induced by immunization with 4 × M2e.HSP70c failed to prevent weight loss in challenged mice, but they experienced significantly lower weight loss, clinical symptoms and higher lung viral clearance in comparison with protective effects of conventional influenza vaccines in challenged mice (Ebrahimi et al., 2012).

To date, the available data to describe immune responses in chicken after the administration of M2e-based vaccine candidates are very limited. However, it was shown that salmonella-vectored vaccine expressing M2e in association with CD154 (CD40 ligand) protected chickens against low pathogenic AI (Layton et al., 2009).

Hence, in agreement with the above mentioned findings, this article describes for the first time the use of r4M2e/H70c fusion protein expressed in *E. coli* to vaccinate chickens, a target host of avian influenza in a manner of case-control study. Furthermore, the humeral and cell-mediated responses elicited against r4M2e/H70c was analyzed with regard to antibodies and cytokines

production. Virus challenge experiments were also assayed to evaluate the protective efficacy of r4M2e/H70c against heterosubtypic avian influenza virus, H9N2, in chicken lungs.

2. Material and methods

2.1. Virus

As previously described (Ebrahimi et al., 2011), the challenge strain was influenza A/Chicken/Iran/339/2002 H9N2 low pathogenic isolated from a commercial layer farm at 53 wk of age, showing severe influenza clinical signs including respiratory distress, swelling of the infraorbital sinuses, and conjunctivitis accompanied with 100% morbidity and high mortality. There was also a 60% to 80% decrease in egg production, along with misshapen, whitish, and fragile eggs.

Avian influenza H9N2 virus strain as the challenge inoculum was passed one additional time in the allantoic sacs of specific pathogen-free (SPF) fertile chicken eggs and then calculated as a median embryo infectious dose (EID50) per milliliter by the Reed and Muench method (Reed and Muench, 1938). Clarified allantoic fluids were aliquoted and then stored at −70 °C.

2.2. Chickens

All experimental procedures were carried out in accordance with the guidelines and with the approval of the local institutional animal experiment committee (Animal rearing Dept., Baqiyatallah University of Medical Sciences, Tehran, Iran). One-day-old broiler chickens (commercial name Ross) were acquired from a local supplier (Amol Joojeh Co., Iran). After 2 weeks in the brooders with free access to water and a standard starter diet, the chickens were divided into five 25-bird groups, bled for baseline serology, transferred to separate location where they were vaccinated and kept until the end of the experimental period.

Before the experiments, all animals were confirmed to be free of avian influenza subtype H9 antibody by the hemagglutination inhibition (HI) test. After challenge, birds were examined daily for clinical signs of disease.

2.3. Vaccines and immunization

Recombinant 4 × M2e (r4Me2) and 4 × M2e.HSP70c fusion protein (r4M2e/H70c) with the M2e consensus sequence of human influenza A virus (H1N1, H2N1, H3N2; amino acid sequence: SLLTEVETPIRNEWGCRCNDSSD) was expressed, purified and prepared as a vaccine candidate from an *E. coli* expression system as previously described (Ebrahimi et al., 2012).

The purified recombinant proteins were stored in F105 buffer which contains 10 mM Tris, 10 mM histidine, 5% (w/v) sucrose, 75 mM NaCl, 0.1 mM EDTA, 0.5% (v/v) ethanol and 0.02%(w/v) polysorbate-80 (pH 7.2) at 4 °C until use.

The mixture contained 30 μg of r4M2e/H70c or r4M2e per dose (0.5 ml/bird) and was used as recombinant M2e-based influenza vaccines.

Avian influenza H9N2 virus strain A/Chicken/Iran/101/1998 (Iran/H9N2) was kindly provided by the Marand branch of Iran's Razi Vaccine and Serum research Institute. Virus was inoculated into the allantoic cavities of 10-day-old embryonated eggs and was harvested after 72 h incubation at 35 °C. The AIV titer of the inoculated allantoic fluid was $10^{9.3}$ 50% egg infectious dose per milliliter (EID_{50}/ml). Then, the virus was inactivated by adding 0.2% formalin (v/v) and kept at 37 °C for 24 h. Inactivation was confirmed by the absence of detectable infectivity after two blind passages of formalin treated allantoic fluid in embryonated eggs. The inactivated allantoic fluid was adjuvanted with MF59 (Novartis' proprietary squalene based oil-in-water nano-emulsion) in a volume ratio 50:50 and was used as a conventional HA-based influenza vaccine (Con/HA) for the following experiments. Allantoic fluid containing infectious virus was also used as an antigen source for HI assay.

Chickens were randomly divided into 5 treatment groups of 25 birds each (Table 1) and tagged for individual identification.

All birds in the appropriate groups as shown in Table 1 were each immunized twice (days 10 and 20) intramuscularly (IM) in the pectoral muscle with a dose of 0.5 ml. Birds of group NC as an uninfected control was kept in the separate room.

2.4. Virus challenge experiment and sampling

Ten days after the final immunization (day 30), chickens were challenged by intra-nasal inoculation of A/Chicken/Iran/339/2002 H9N2 low pathogenic at a dose of $10^{6.8}$ EID_{50} in 100 μl of PBS per bird. Challenged chickens were monitored daily for 14 days after infection for the apparition of clinical signs (reluctance to move, anorexia, congestion of eyes, respiratory signs mainly sneezing, and swollen head). Body weight gains were also measured between days 30 and 44.

Table 1
Definitions of experimental groups.

Group	No. of immunized birds	Definition
NC	25	Negative control group given F105 buffer intramuscularly at 10 and 20 days of age
CC	25	Challenge control group given F105 buffer intramuscularly at 10 and 20 days of age
Con/HA	25	Group given conventional HA-based influenza vaccine intramuscularly at 10 and 20 days of age
r4M2e/H70c	25	Group given 30 μg/bird of recombinant 4 × M2e.HSP70c fusion protein formulated with F105 buffer intramuscularly at 10 and 20 days of age
r4M2e	25	Group given 30 μg/bird of recombinant 4 × M2e fusion protein (without HSP70c) formulated with F105 buffer intramuscularly at 10 and 20 days of age

Blood samples were taken from challenged birds on days 7 and 14 after infection for HA-antibody titration using hemagglutination inhibition (HI) technique.

Protection was expressed as the percentage of live and healthy birds at 14 days post-challenge.

Oropharyngeal and cloacal swabs were collected on days 2, 4, and 7 post-challenges from 5 challenged birds in each group to evaluate virus shed titers. These time points represent the peak in oropharyngeal and cloacal shedding, respectively (Spackman et al., 2010; Le Gall-Reculé et al., 2007).

Swab tips were transferred to 1 ml viral transport medium containing antibiotics and antimycotics compounds (Gibco, Grand Island, NY, USA), vortexed briefly and medium was either frozen at −80 °C.

2.5. Virus titration from swabs

Virus titers were determined as the 50% tissue culture infectious dose ($TCID_{50}$) as described previously (Chen et al., 2007; Qiu et al., 2006). MDCK cells (a kindly gift from Dr. Mirjalili, Razi Vaccine and Serum Research Institute) were inoculated with a 10-fold serial dilution of sample and incubated at 37 °C in a humid atmosphere of 5% CO_2 for 1 h. After 1 h of absorption, media was removed and overlay medium containing L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Thermo Fisher Scientific, Rockford, USA) was added to the infected cells and incubated for 3 days. Viral cytopathic effects were observed daily, and titers were determined by the HA test. For HA, chicken red blood cells (0.5%) were added to 50 μl of cell supernatant and incubated for 30 min. The virus titer of each sample was expressed as 50% tissue infected doses using the Reed-Muench method.

2.6. Serological analysis

Pre-vaccination sera were collected from all vaccinated chickens. Five chickens were sampled randomly from each group on days 20 and 30 after immunization and blood samples were collected via wing vein puncture. Sera at a 1:400 dilution prepared in PBS were tested for development of M2e- or HA-specific antibodies.

Serum samples, five birds from each group, were also collected from the surviving birds on days 7 and 14 post-challenge for detection of HA specific antibody responses. Serum samples from all groups and all time points were heat inactivated at 56 °C for 30 min before being analyzed in ELISA or hemagglutinin inhibition (HI) assays for analysis of anti-M2e or HA-antibody responses, respectively. All negative samples for the HI test were set at 2 $\log_2$, all negative samples for the ELISA test were set at 0.5 OD, and all negative samples for the virus titration test were set at 0.9 $\log_{10}$ EID_{50}/ml.

The HI assay was carried out according to our previous study (Ebrahimi et al., 2011) using formalin-treated Iran/H9N2 antigen and 1% v/v suspension of chicken red blood cells (RBCs). All samples were analyzed on the same day, and HI titers were scored as the reciprocal of the highest serum dilution producing 50% inhibition of hemagglutination.

The ELISA assay was performed as described previously (Ebrahimi et al., 2012). Briefly, 96 well plates (Maxisorb, Nunc, Denmark) were coated either with 1 μg/ml of synthetic M2e-peptide (23 amino acids, 2 to 24, SLLTEVETPIRNEWGCRCNDSSD, synthesized by solid phase technology at the GL Biochem, China), in 50 mM sodium bicarbonate buffer, pH 9.6, and incubated overnight at 4 °C.

After blocking, different serum samples were loaded on peptide coated plates. The plates were incubated with the different serum samples and then incubated with 1:10000 goat anti-chicken IgG-HRP conjugates (Sigma-Aldrich, USA), for 1 h at 37 °C. The color reaction was developed with 3,3′,5,5′-tetramethylbenzidine, TMB, (Pishtazteb, Tehran, Iran), at OD 450 nm. For comparison between groups, the averages of A450 values of different sera were analyzed. The antibody titer is defined as the reciprocal of the highest dilution that yields an OD 450 nm value above 2 times the mean of negative control wells.

2.7. Lymphocyte proliferation and immunophenotyping assays

An antigen specific lymphocyte proliferation assay was performed using a modified MTT method as described previously (Kong et al., 2004; Mosmann, 1983). Ten days after the final immunization (day 30), blood samples from 5 birds each group which were chosen for serological assay on day 30 after immunization were collected for lymphocyte separation via wing vein puncture in 2.5 ml syringes preloaded with 0.2 ml sodium heparin.

The cell suspension from the blood was layered on Ficoll-Paque lymphocyte separation medium by density gradient centrifugation. Peripheral blood lymphocytes (PBLs) were obtained from the interface and washed twice with Hanks' balanced salt solution. After centrifugation, the final pellet was resuspended in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 5% heat-inactivated fetal calf serum at a concentration of 2×10^6 cells per ml and seeded in a 96-well plate in triplicate (3×10^5 cells per well).

Cultures were stimulated under various conditions included treatment with 5 μ/ml concanavalin A (Sigma-Aldrich, USA) as a positive control, 5 μg/ml synthetic M2e-peptide as a specific antigen, 5 μ/ml bovine serum albumin, BSA, (Sigma-Aldrich, USA) as an irrelevant antigen or RPMI 1640 medium without antigen as a negative control.

After 60 h of incubation at 37 °C in a humid atmosphere with 5% CO_2, 20 μl of MTT (Sigma-Aldrich, St. Louis, MO) was added to each well and the incubation was continued for another 4 h. Then 100 μl of dimethyl sulfoxide (DMSO) was added, and incubation was continued for an additional 24 h before measurement of OD at 570 nm (OD570) using an ELISA reader (Bio-Tek Instruments, VT). The stimulation index (S.I.) was calculated as the ratio of the average OD of antigen-stimulated cells to the average OD of unstimulated cells.

For examination of changes in T cell populations, 100 μl of PBLs isolated as described above was incubated for 1 h at 4̊C with the following antibodies (double labelling): monoclonal antibody phycoerythrin (PE)-labeled anti-chicken $CD3^+$ and then with PE-labeled anti-chicken

$CD4^+$ or fluorescein isothiocyanate (FITC)-conjugated anti-chicken $CD8^+$ (Southern Biotechnology, USA).

PE- and FITC-conjugated isotype controls were also included. Cells were analyzed by fluorescence-activated cell sorting (BD Biosciences).

2.8. Cytokine assays

Aliquots of the same PBLs samples used to set up the lymphocyte proliferation assay were tested for the production of IFN-γ and IL-4 cytokines with commercial chicken IFN-γ and IL-4 sandwich ELISA kits (Biosource, China).

Sterile 96 well round bottom plates at 4×10^5 cells per well in triplicate containing 5 μg/ml synthetic M2e-peptide was used in the assay. Control wells were prepared by adding 500 ml complete RPMI alone (negative control).

For the IFN-γ and IL-4 detection, plates were centrifuged for 10 min at $600 \times g$ after 48 h incubation at 37 °C in a CO_2 incubator. Supernatants from the spun plates were collected and then analyzed for detection of chicken Th1-type cytokine (IFN-γ) and Th2-type cytokine (IL-4) secretion as described in kit instruction manual.

Briefly, standards or samples were added to the appropriate pre-coated plate wells with Horseradish Peroxidase (HRP) conjugated antibody preparation specific for IFN-gamma or IL-4. The competitive inhibition reaction was launched between pre-coated antigens and appropriate cytokines in samples. A substrate solution was added to the wells and the color develops in opposite to the amount of cytokines in the samples. The color development was stopped and the intensity of the color was measured. The concentrations of cytokines secretion in the samples were determined from the standard curves.

2.9. Statistical analysis

Data were analyzed using the SPSS 13.0 software (SPSS Inc., Chicago, USA). Data were analyzed for significance ($P < 0.05$) by the one-way analyses of variance when variance between groups was homogeneous and distribution of the data was normal or a nonparametric test (Kruskal–Wallis) when normality test or homogeneity of variance test failed.

3. Result

3.1. Serologic response to immunization

The analysis of pre-vaccination swabs and serum samples by virus isolation and HI tests revealed that all birds used in this study had no detectable infection with H9 viruses and the levels of HI antibody titers were $<2 \log_2$ against these virus subtypes (data not shown). The uninfected control groups remained healthy throughout the experiment and showed no clinical disease signs. H9N2-specific HI titers remained at baseline values ($\leq 2 \log_2$) in the serum samples recovered from this control group just before termination of the experiments.

Serum from chickens immunized with M2e-based vaccines or HA-based vaccine were analyzed by ELISA and HI assays to identify antibodies directed against M2e peptide or HA glycoprotein of influenza A isolates, respectively.

All birds initially immunized and subsequently boosted with M2e-based vaccine or conventional vaccines containing HA had an anti-M2e (Fig. 1) and anti-HA (Fig. 2) antibody responses as measured by ELISA and HI assays, respectively.

No anti-M2e antibody was detected in birds immunized with Con/HA (Fig. 1). There was statistically significant difference in induced anti-M2e antibodies among M2e-based immunized groups of birds only after boosting, in that birds immunized with r4M2e/H70c recombinant fusion protein showed higher titer of anti-M2e antibody than observed with r4M2e (Fig. 1; $P < 0.05$). The results suggested that mHSP70c could induce enhanced humoral responses against the M2e protein.

No anti-HA antibody was produced in birds immunized with r4M2e/H70c as measured by HI assay, prior to viral challenge. However, the mean titers of antibodies enhancement with HA glycoprotein were detected in birds vaccinated with r4M2e/H70c or r4M2e or Con/HA on days 7 and 14 after challenges by using homologous formalin-treated viruses antigen (Fig. 2). These data confirm that inactivated conventional HA-based vaccine or M2e-based vaccines could not fully prevent infection in birds. However, r4M2e/H70c fusion protein as a vaccine candidate significantly decreased viral shedding and clinical signs in challenged birds (Tables 2 and 3).

Table 2
Protective efficacy of prime—boost strategies in chickens against H9N2 virus challenge.

Group	Virus isolation from swabs on different days p.c.: no. of shedding birds/total (mean titers [$\log_{10}$ $TCID_{50}$/ml] ±S.E.M)					
	Day 2		Day 4		Day 7	
	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
NC	0/25 (0)	0/25 (0)	0/25 (0)	0/25 (0)	0/25 (0)	0/25 (0)
CC	25/25 (4.9 ± 0.5)*	12/25 (3.4 ± 0.5)	25/25 (6.2 ± 0.6)	22/25 (5.5 ± 0.4)	10/25 (4.2 ± 0.4)	5/25 (2.7 ± 0.1)
Con/HA	25/25 (3.6 ± 0.3)	8/25 (2.4 ± 0.4)	25/25 (4.7 ± 0.4)	17/25 (4.3 ± 0.3)	11/25 (3.3 ± 0.3)	0/25 (0)
r4M2e/H70c	25/25 (4.1 ± 0.2)	11/25 (2.5 ± 0.2)	25/25 (3.6 ± 0.2)	15/25 (3.5 ± 0.2)	0/25 (0)	0/25 (0)
r4M2e	25/25 (4.4 ± 0.4)	11/25 (2.7 ± 0.2)	25/25 (5.4 ± 0.4)	19/25 (4.5 ± 0.3)	14/25 (3.5 ± 0.3)	0/25 (0)

Note: Definition of each group is given in Table 1.

* Numbers show $\log_{10}$ geometric mean $TCID_{50}$/ml ±standard error of the mean (S.E.M) of five birds/group per time point.

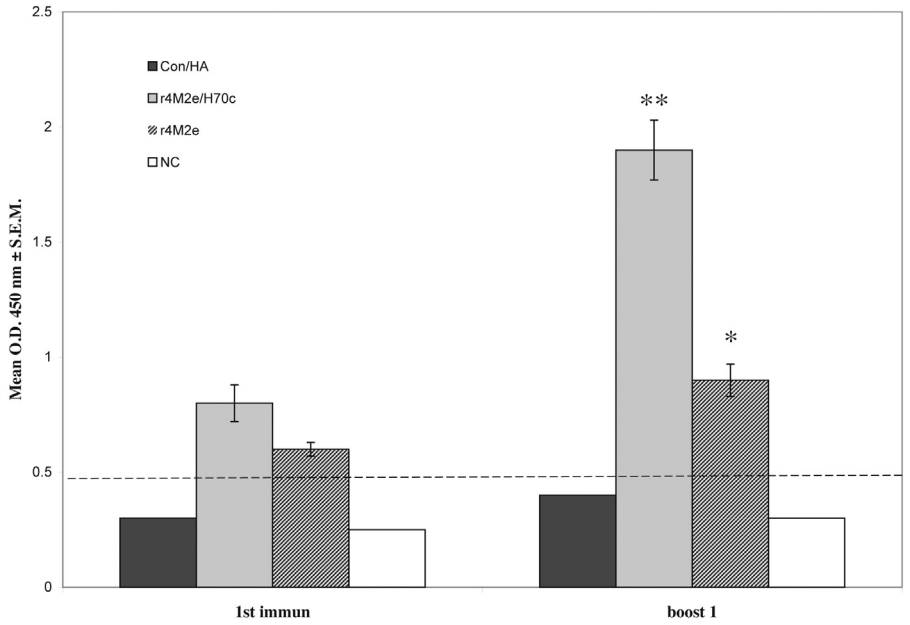

Fig. 1. M2e specific antibodies (1:400 diluted in PBS) were determined by ELISA. 96-well plates were coated with peptide M2e. Anti-sera were collected 13 days after the first immunization (1st immun) and the second immunization (boost 1), respectively, from mice immunized with 4 × M2e.HSP70c administrated in F105 buffer. The bound antibodies were detected by horseradish peroxidase-linked rabbit-anti-mouse antibodies and TMB solution. Mean value ± S.E.M of A450 in each group were presented. The titers of antibodies were expressed as the highest dilution of a serum which has twice average value of A450 in control group. Bars show mean value ± standard error of the mean (S.E.M) of five birds/group per time point. The dashed line shows the limit of detection. Statistically significant differences ($P < 0.05$) are indicated by * (compared with NC or Con/HA) or ** (compared with r4M2e alone).

3.2. Clinical signs, mortality and virus shedding

To determine and compare the protective efficacy among different vaccinated groups, all of the immunized chickens were challenged intranasally with $10^{6.8}$ EID50/0.1 ml of H9N2 AIV. Clinical signs, protection rates and viral shedding after challenge of chickens are summarized in Tables 2 and 3.

In all experimental groups, protective efficacy among immunized chickens following challenge was monitored

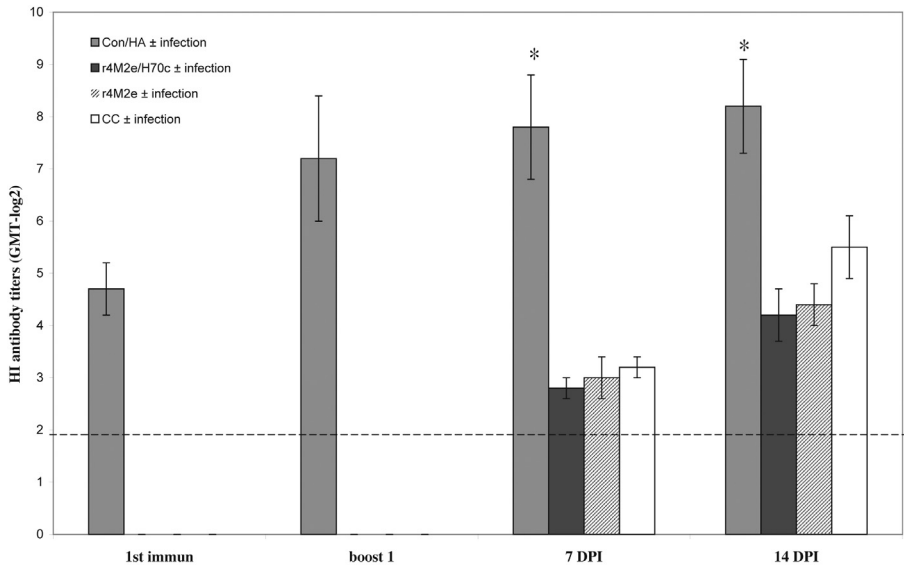

Fig. 2. Mean hemagglutinin-inhibition (HI) antibody titers from birds immunized with Con/HA, r4M2e, and r4M2e/H70c 10 days after the first immunization (1st immun) and the second immunization (boost 1), respectively. Formalin-treated Iran/H9N2 antigen was used in HI assay for analysis of anti HA-antibody responses induced by conventional vaccines. The mean titers of antibodies enhancement with HA glycoprotein were detected in birds vaccinated with r4M2e or r4M2e/H70c on 7 and 14 day post infections (7DPI and 14DPI) by using homologous formalin-treated Iran/H9N2 antigen. Bars show log 2 geometric mean ± standard error of the mean (S.E.M) of five birds/group per time point. The dashed line shows the limit of detection. Statistically significant differences ($P < 0.05$) are indicated by * (compared with other groups on 7 and 14 day post infections). (±Infection) indicates as before (−) or after (+) infection.

Table 3
Clinical signs and mortality in immunized chickens after challenge with LPAI H9N2 virus.

Group	No. of challenged birds	Clinical signs		Mortality
		No. of sick birds/total (%)	BW (g ± S.D.)*	No. of dead birds/total (%)
NC	25	0/25 (0)	710 ± 2 2 a	0/25 (0)
CC	25	25/25 (100)	460 ± 77 b	6/25 (24)
Con/HA	25	23/25 (92)	545 ± 43 c	0/25 (0)
r4M2e/H70c	25	25/25 (100)	670 ± 14 a	0/25 (0)
r4M2e	25	25/25 (100)	510 ± 35 c	3/25 (12)

Data within BW column with different letters are significantly different ($P < 0.05$).
* Body weight was measured 14 days post-challenge (p.c.).

by measuring body weight and observation of clinical symptoms.

From day 3 after viral challenges, the challenged and non vaccinated birds (CC group) developed obvious clinical signs including: general depression, sneezing, respiratory sounds, ruffled feathers, and eye redness. Clinical signs were observed from 2 to 8 days post-challenge and birds were quiet and reluctant to move.

These clinical signs were delayed for 2 days in the birds vaccinated with the r4M2e/H70c, r4M2e or Con/HA, in which they exhibited mild clinical signs for 2–3 days.

By day 5 post-challenge, the birds immunized with r4M2e/H70c showed some recovery. On day 7 post-challenge, they were behaving normally and their activity and appetite returned to normal levels.

As shown in Table 3, the birds immunized with r4M2e/H70c showed weight loss following the viral challenge; however, this was less severe than that observed for the challenged non-vaccinated birds (group CC) and birds vaccinated by Con/HA or r4M2e vaccines. The difference in mean body weight loss of r4M2e/H70c immunized group of birds following viral challenges with compared to other groups of challenged birds was also highly significant (Table 3; $P < 0.05$). There was no significant difference in mean body weight loss between r4M2e/H70c immunized group of birds and unchallenged birds (NC group). It is concluded that weight loss is associated with the viral load of H9N2 virus in birds.

None of the chickens immunized with r4M2e/H70c or Con/HA vaccines died after challenge, whereas six of the 25 chickens injected with the F105 buffer (CC group) and three of the 25 chickens immunized with r4M2e died from the disease.

To measure virus shedding, oropharyngeal and cloacal swabs were obtained from all chickens on days 2, 4 and 7 post-challenge and assessed by virus titration in MDCK cells. As shown in Table 2, no virus was isolated from the oropharyngeal and cloacal swabs of the unchallenged birds (NC group). All challenged birds showed respiratory viral replication at days 2, and 4 after challenges with LPAI H9N2 virus. The birds immunized with r4M2e/H70c had overall significantly lower oropharyngeal and cloacal viral shedding than did the challenged CC, r4M2e or Con/HA vaccinated birds (Table 2; $p < 0.05$).

At day 7 post-challenge, no viruses were detected in the birds immunized with r4M2e/H70c; whereas, viruses were

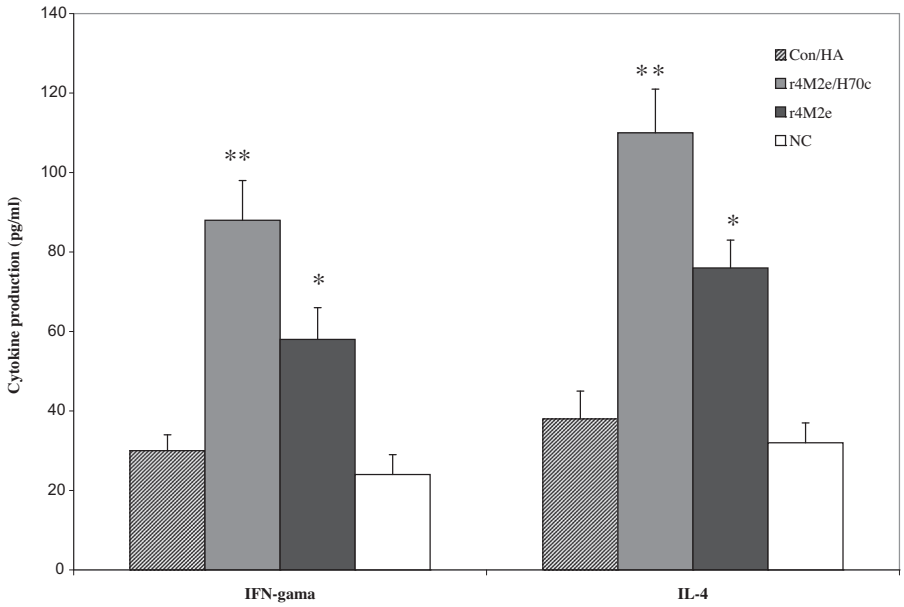

Fig. 3. Levels of cytokine production from PBLs after M2e stimulation *in vitro*. The test was repeated five times and values are expressed as mean ± S.E.M. Statistically significant differences ($P < 0.05$) are indicated by * (compared with NC or Con/HA) or ** (compared with r4M2e alone).

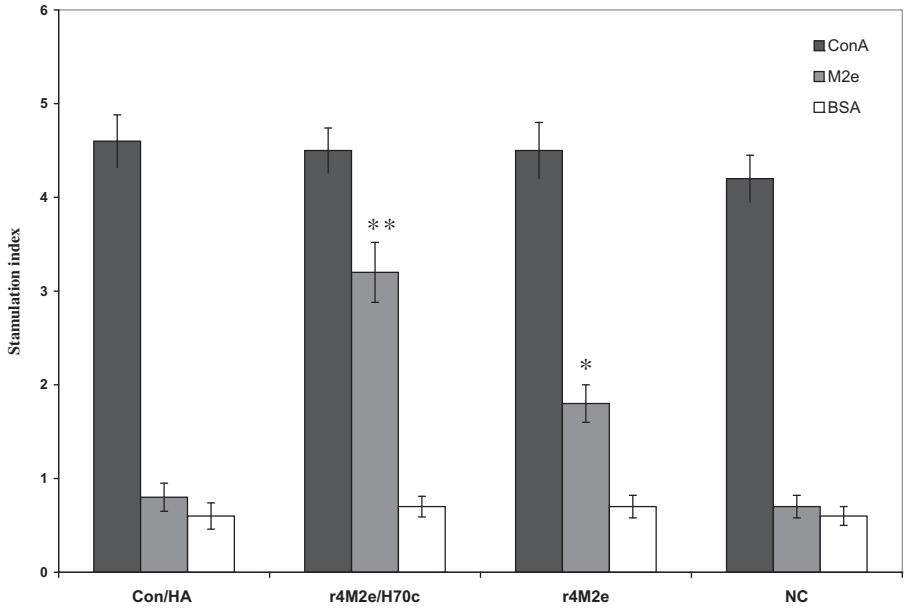

Fig. 4. Peripheral blood T-lymphocyte proliferation assay. The test was repeated three times and values are expressed as mean ± S.E.M. Statistically significant differences ($P < 0.05$) are indicated by * (compared with NC or Con/HA) or ** (compared with r4M2e alone). ConA, Concanavalin A.

detected in the other challenged birds. With regard to periods of viral shedding, the birds that received r4M2e/H70c had lower periods of virus replication.

3.3. Levels of Th1 and Th2 cytokines

We investigated the effect of r4M2e with and without HSP70c on changes in Th1 and Th2 phenotypes. As shown in Fig. 3, the increase in the production of IFN-γ (Th1-type) and IL-4 (Th2-type) after r4M2e/H70c was significantly higher than that after injection of r4M2e alone when stimulated with synthetic M2e peptide, whereas the chickens vaccinated with con/HA or F105 buffer did not respond to the M2e peptide ($P < 0.05$). This profile of cytokine secretion suggested that mHSP70c enhances the induction of immune responses by promoting a Th1-dominant response.

3.4. Lymphocyte proliferation assay

The cellular immune response was examined by determining the ability of chicken peripheral mononuclear cells to show a proliferative response against synthetic M2e peptide. As shown in Fig. 4, an enhanced T-cell proliferative response to M2e peptide was clearly observed in the groups immunized with r4M2e/H70c or r4M2e when stimulated with synthetic M2e peptide, whereas the chickens vaccinated with con/HA or F105 buffer did not respond to the M2e peptide.

The level of the T-cell proliferative response in the group immunized with r4M2e/H70c was significantly higher than that in the group immunized with r4M2e ($P < 0.05$). Chickens in all groups responded similarly to stimulation with ConA ($P > 0.05$).

These results indicated that higher levels of M2e-specific T-cell proliferative responses could be elicited by immunization with r4M2e/H70c fusion protein than by immunization with r4M2e alone.

3.5. Immunophenotyping of peripheral T cells

As $CD4^+$ and $CD8^+$ T-lymphocytes are among the most crucial components of antiviral effectors, these lymphocytes were assessed in immunized chickens. Flow cytometric analysis of unstimulated cells was used to standardize the background responses. As shown in Table 4, immunization with M2e-based vaccine (r4M2e/H70c or r4M2e) or HA-based vaccine (Con/HA) significantly increased the percentages of $CD4^+CD3^+$ and $CD8^+CD3^+$ T-lymphocytes compared

Table 4
Ratios of $CD4^+$ to $CD8^+$ T lymphocytes after final vaccination.

Group	Week post vaccination			
	1	2	3	4
Con/HA	1.84 ± 0.25 a	1.81 ± 0.21 a	1.79 ± 0.15 a	1.80 ± 0.09 a
r4M2e/H70c	2.08 ± 015 b	2.17 ± 0.11 b	2.05 ± 0.23 b	2.02 ± 0.21 b
r4M2e	1.66 ± 0.14 c	1.65 ± 0.08 c	1.63 ± 0.12 c	1.59 ± 0.15 c
NC	1.46 ± 0.13 d	1.45 ± 0.09 d	1.42 ± 0.22 d	1.38 ± 0.15 d

Data are expressed as means ± S.E.M. Data are presented as percentage of total cells.
Data within a column with different letters are significantly different ($P < 0.05$).

with the percentages in the chickens immunized with F105 buffer ($P < 0.05$).

There was a significant difference in the percentages of $CD4^+CD3^+$ and $CD8^+CD3^+$ T-lymphocytes between the group immunized with r4M2e/H70c and the group immunized with r4M2e or Con/HA ($P < 0.05$). The ratios of $CD4^+$ to $CD8^+$ lymphocytes in r4M2e/H70c vaccinated group was significantly higher than in groups immunized with r4M2e or Con/HA from the first week after vaccination (Table 4, $P < 0.05$). These results showed that the r4M2e/H70c can elicit a cellular immune response in chickens significantly.

4. Discussion

Avian influenza virus (AIV) is one of the most important respiratory pathogens which annually pose significant public health concern and devastating economic losses to the commercial poultry, especially chickens industry, worldwide (Alexander, 2003; Senne, 2003).

Up to now, vaccination has played an important role in the protection of chickens against AIV infections. Currently available influenza vaccine used in chickens is inactivated whole virion that only induce strong neutralizing antibody specific for the highly variable surface hemagglutinin (HA) glycoprotein, and it is commonly accepted that no adequate cellular immunity is achieved (Moscona, 2005; Wareing and Tannock, 2001).

Neutralizing antibodies exert strong immune pressure on influenza virus to mutate and reassort continuously and thus could escape from vaccine protection, leaving hosts only partially protected against the next influenza infection (Sun et al., 2012). Thus, cellular immunity is essential for virus clearance and an improved AIV vaccine is needed to induce complete immunity against drift variants within a subtype of AIVs in poultry industry.

This study was conducted to evaluate the immunogenicity and protective efficacy of an *E. coli*-expressed 4 × M2e.HSP70c fusion protein comprising C-terminus of *Mycobacterium tuberculosis* HSP70 (mHSP70c) genetically fused to four tandem repeats of the ectodomain of the conserved influenza matrix protein M2 (M2e) using chicken as the target host for AIVs.

The aim of universal influenza vaccination approaches is to provide long-lasting protection against a wide range of viral serotypes. Hence, M2e protein-based vaccine approaches represent one such vaccine as its N-terminal epitope SLLTEVET (residues 2–9) found to be conserved at a rate of over 99.3% among all subtypes of influenza A viruses (Fiers et al., 2004; Liu et al., 2005).

The reason of linking M2e to adjuvant portion of mHSP70 is that the targeting of antigen to APCs through HSP70 receptors such as CD91 is a useful strategy to induce CD8 T cell and antibody responses by alternate MHC-I Ag processing (cross-processing) mechanisms, resulting in cross-presentation of the exogenous antigens to CD8T cells that are crucial to immune responses against viruses (Arnold et al., 1995; Blachere et al., 1997; Tobian et al., 2004). Moreover, Hsp70 can also activate the innate immunity through CD40 and Toll-like receptor-2 (TLR-2) and TLR-4 to induce cytokine secretion (Takakura et al., 2007; Takemoto et al., 2005).

Our previous study showed that a prime-boost administration of 4 × M2e.HSP70c (r4M2e/H70c) formulated in F105 buffer provided full protection against lethal dose of mouse-adapted H1N1, H3N2, or H9N2 influenza A viruses in mice (Ebrahimi et al., 2012). In addition, Oral live attenuated *Salmonella Enteritidis* vaccine vector expressing M2e protein of influenza A virus in combination with CD154 without adjuvant effectively induced M2e-dpecific IgG antibody response in chicken and provided protection against direct challenge with LPAI H7N2 (Layton et al., 2009).

In the present study, 30 μg of r4M2e/H70c without adjuvant compared to HA-based vaccine formulated with MF59 adjuvant enhanced humoral and cell-mediated immune responses in chickens. In addition, increase of HI antibody titers against H9N2 virus in all M2e- or HA-based vaccinated birds after challenge indicated that the challenge virus replicated in all immunized groups.

Oropharyngeal and cloacal shedding of the virus was detected in all of the r4M2e/H70c vaccinated birds at 2 days after challenge, but the titer was low and decreased rapidly to reach undetectable levels at 7 days after challenge. These results agreed with the clinical signs observed and suggest higher resistance of the birds vaccinated with r4M2e/H70c to the challenge strain. This result not only exactly agreed with our previous study in mice (Ebrahimi et al., 2012), but it was generally in accordance with the previous reports on M2e-based vaccines in mice (Wu et al., 2007; Ernst et al., 2006; Sealy et al., 2003; Huleatt et al., 2008).

Anti-M2e antibodies are not neutralizing antibodies; however, they are capable of binding to the M2 proteins that are abundantly presented on the surface of the infected cells to eliminate influenza virus-infected cells through antibody-dependent cell-mediated cytotoxicity (ADCC) and/or phagocytosis (Jegerlehner et al., 2004; Tompkins et al., 2007). Furthermore, they are predicted to limit the severity of influenza A disease by its inhibitory role on the uncoating of viral particles in endosomes (Betakova, 2007). As a result, the induced anti-M2e antibodies can delay the onset of morbidity and allow the host immune response to develop adaptive immunity to the dominant neutralizing influenza antigen, HA. Therefore, humoral immune responses against HA provoked by infection could help to reduce viral replication in the respiratory system.

This explanation can be considered not only by the results of our previous study in mice (Ebrahimi et al., 2012), but in the present study induced HA-specific serum antibody was detected by day 7 after challenge (Fig. 2). Other published studies also reported the detection of HA-specific serum antibody by day 5 to 6 after challenges with significant impact to restrict viral replication in the respiratory system (Fabrice et al., 2008; Lincoln et al., 2010; Sealy et al., 2003).

With regard to the above explanation and considering that the replication of the challenge influenza virus can induce a significant increase in the HA-specific serum antibody titers and the absence of this antibody increase suggests that the challenge virus did not replicate to a significant degree. Consequently, it seems that the recovery of r4M2e/H70c vaccinated birds with the low level of

HA-specific antibody (Fig. 2) by day 5 after challenge is likely contributed to induced HA-subtype specific neutralizing antibody.

Clinical signs and shedding levels could not be used here as the only criteria to compare the efficacy of the different vaccine schemes. Therefore, another way to evaluate indirectly the efficacy of vaccines was to compare the activation and proliferation of lymphocytes as a critical role in inducing both the humoral and cellular immune responses after immunization.

Our results showed that the T cells of chickens immunized with r4M2e/H70c or r4M2e in the absence of any adjuvant exhibited a proliferative response. However, the level of M2e-specific T-cell proliferative responses in the chickens immunized with r4M2e/H70c was significantly higher than that in the chickens immunized with the r4M2e vaccine. These data clearly showed that mHSP70c is a strong adjuvant that modulates the immune response toward the induction of strong cellular response with a Th-1 cytokine profile.

In this study, protection against H9N2 viral challenge was significantly increased in chickens by injection of r4M2e/H70c compared with injection of Con/HA or r4M2e. This protective immunity might be attributed to enhanced cell-mediated immunity, which is interpreted as increased lymphocytes proliferation, increased levels of Th1-type (IFN-γ) and Th2-type (IL-4) cytokines production and increased $CD4^+$ to $CD8^+$ ratios, resulting from the injection of r4M2e genetically fused to mHSP70c. These results are consistent with previous reports on antibodies and cell-mediated cytotoxicity specific to the M2e antigen fused to ASP-1 or in combination with nucleotide based CPG-ODN adjuvant and their anti-viral activity (Wu et al., 2009; Zhao et al., 2010).

In Conclusion, this is the first report of a challenge study evaluating the immunogenicity and protective efficacy of an *E. coli*-expressed 4 × M2e.HSP70c formulated in F105 buffer as a universal influenza vaccine candidate by a prime-boost vaccination regime against influenza virus in chickens. As, protective immune responses against internal pathogens like viruses are known to require a balance between Th-2 and Th-1 response patterns with the production of type 1 cytokines such as IFN-γ and antibodies of the IgG2a isotype in mice (Stevens et al., 1988; Unkeless et al., 1988). Accordingly, our data reported herein show that vaccination with r4M2e/H70c promoted a cellular immune response characterized by increased levels of Th1-type (IFN-γ) and Th2-type (IL-4) cytokines production and increased $CD4^+$ to $CD8^+$ ratios.

Moreover, comparison of protective efficacy against LPAI H9N2 in birds intramuscularly immunized with H9N2/Iran vaccine or r4M2e/H70c fusion protein likely represented the ability of the M2e-based vaccine in providing cross-protection against heterosubtypic H9N2 challenge and also allowed the host immune system to induce HA-homosubtype neutralizing antibody against H9N2 challenge. Consistently, HA-specific neutralizing antibody was reported herein to have sub-typically significant impact on influenza virus replication by day 5 after challenge, these result is in accordance with previously described study (Furuya et al., 2010), in which the cross-protection was contributed to influenza-specific CTL activity (cell-mediated immunity).

Taking to gather, the M2e based-vaccine could not prevent the viral infection but rather suppresses the symptoms of virus-infected chickens by reducing the virus shedding in chicken. It will be worthwhile to evaluate the immune response further in the chicken model with other formulation of r4M2e/H70c by different routs of injection.

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