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Research paper

A DNA prime-protein boost vaccination strategy targeting turkey coronavirus spike protein fragment containing neutralizing epitope against infectious challenge

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ABSTRACT

The present study was undertaken to determine immune response and protection efficacy of a spike (S) protein fragment containing neutralizing epitopes (4F/4R) of turkey coronavirus (TCoV) by priming with DNA vaccine and boosting with the recombinant protein from the corresponding DNA vaccine gene segment. Turkeys were vaccinated by priming with either one dose (G1-750DP) or two doses (G3-750DDP) of 750 μg DNA vaccine expressing 4F/4R S fragment and boosting with one dose of 200 μg 4F/4R S fragment. One dose of 100 μg DNA vaccine mixed with polyethyleneimine (PEI) and sodium hyaluronate (HA) followed by one dose of 750 μg DNA vaccine and one dose of 200 μg 4F/4R S fragment were given to the turkeys in group G2-100DPH. After infectious challenge by TCoV, clinical signs and TCoV detected by immunofluorescence antibody (IFA) assay were observed in less number of turkeys in vaccination groups than that in challenge control groups. TCoV viral RNA loads measured by quantitative real-time reverse transcription-PCR were lower in vaccinated turkeys than those in challenge control turkeys. The turkeys in G3-750DDP produced the highest level of TCoV S protein-specific antibody and virus neutralization (VN) titer. Comparing to the turkeys in G1-750DP, significantly less TCoV were detected by IFA in the turkeys in G2-100DPH receiving an extra dose of 100 μg DNA mixed with PEI and HA. The results indicated that DNA-prime protein-boost DNA vaccination regimen targeting TCoV S fragment encompassing neutralizing epitopes induced humoral immune response and partially protected turkeys against infectious challenge by TCoV.

Abbreviations: ANOVA, one-way analysis of variance; CFA, complete Freund's adjuvant; CLPEI, disulfide-crosslinked low molecular linear polyethyleneimine; CpG, cytosine-phosphate-guanine; Ct, threshold cycle value; DPH, DNA, polyethyleneimine, and sodium hyaluronate complex; dpi, days post inoculation; E, envelope; *E. coli*, *Escherichia coli*; EID_{50}, median embryo infectious dose; GM-CSF, granulocyte-macrophage colony-stimulating factor; HA, hyaluronate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline; HRP, horseradish peroxidase; IBDV, infectious bursal disease virus; IBV, infectious bronchitis virus; IFA, imnunofluorescent antibody assay; Kb, kilobase; kDa, kilodalton; LB, lysogeny broth; M, membrane; MHV, mouse hepatitis virus; N, nucleocapsid; NTC, non-template control; OD 450 nm, optical density at 450 nm; qRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; S, spike; SARS-CoV, severe acute respiratory syndrome-coronavirus; TCoV, turkey coronavirus; VN, virus neutralization.

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1. Introduction

Turkey coronavirus (TCoV), a member of the family *Coronaviridae* in the order *Nidovirale*, causes atrophic enteritis in infected turkeys leading to poor feed conversion, uneven body weight gain, and significant economic loss in the turkey industry in the United States (Lin et al., 2002), Canada (Dea et al., 1986; Gomaa et al., 2009), Europe (Cavanagh et al., 2001; Maurel et al., 2011), and Brazil (Teixeira et al., 2007). Clinical signs of TCoV infection include watery diarrhea, depression, ruffled feathers, and decreased consumption of food and water. Distended intestines with gaseous and watery contents are the most striking gross lesions. The major histopathological changes are blunted villi, increased crypt depth, and increased width between villi (Guy, 2003; Gomaa et al., 2009). Currently, there are no effective vaccines available to control TCoV infection.

TCoV is an enveloped virus with a liner positive-sense, single-stranded RNA genome with a size of 27 kilobases (Kb). Spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins are four major structure proteins of TCoV (Lin et al., 2002; Cao et al., 2008). The amino-terminal S1 subunit of S protein forms the globular head of the spike structure on the surface of coronaviruses and contains the receptor binding domain and neutralizing epitopes to determine host tropism and humoral immunity (Godet et al., 1994; Taguchi and Kubo, 1995; He et al., 2004; Ho et al., 2004; Lu et al., 2004; Zhou et al., 2004; Chen et al., 2011). The carboxyl-terminus S2 subunit of S protein constructs the stalk of the spike structure and consists of a transmembrane domain and fusion peptides to induce cell fusion and viral assembly (Spiga et al., 2003; Broer et al., 2006; Howard et al., 2008). Immunization by coronaviral S protein-based vaccines can produce protective neutralizing antibodies that prevent infection after challenge (Johnson et al., 2003; Zeng et al., 2004; Du et al., 2009; Shil et al., 2011).

DNA vaccine, a plasmid DNA encoding the protein from the pathogen of interest under the control of a eukaryotic cell promoter has been demonstrated to induce humoral and cellular immune responses (Laddy and Weiner, 2006). Several approaches have been also investigated to improve the low immunogenicity of DNA vaccine (Barouch, 2006), including co-administration of genes encoding the immunostimulatory molecules like IL-2 (Tang et al., 2008), cytosine-phosphate-guanine (CpG) motifs (Kinman et al., 2010), or granulocyte-macrophage stimulating factor (GM-CSF) (Tan et al., 2009) to enhance immune responses. Other approaches to improve immunogenicity of DNA vaccine included targeting DNA toward proper antigen presenting cells like dendritic cells (Kurzler and Weiner, 2004; Nchinda et al., 2008), applying prime-boost strategy by priming with DNA and boosting with a viral vector encoding the antigenic protein (Woo et al., 2005; Wang et al., 2008), and improving DNA delivery by electroporation or incorporation with nanoparticles to increase the amount and stability of DNA inside the transfected cells and thus enhance the protein expression level by transfected DNA (Otten et al., 2005; van Drunen Littel-van den Hunk and Hannaman, 2010).

Nanoparticles, made of cationic lipids and polymers, can protect DNA and deliver DNA to target cells. Polyethyleneimine (PEI) is a widely used polymer and the size of the DNA–PEI complex is suitable for endocytic uptake by non-phagocytic cells (Jeong et al., 2007). Once inside the cells, PEI absorbs proton ions during endosome acidification, leading to osmotic rupture of endosome and DNA release to enter the nucleus of the transfected cells (Boussif et al., 1995). The addition of polysaccharide-based polyanions, like hyaluronate (HA) improved the gene transfection efficiency of the DNA–PEI complex by loosening up the complex to access the gene transcription machinery (Ito et al., 2006). Polycations with disulfide linkages, which could degrade in the reductive intracellular environment and release DNA more easily, achieved higher gene expression than their uncleavable counterparts (Neu et al., 2006). By applying the advantages exerted by HA and disulfide linkage, a ternary complex (DPH complex) consisting of DNA, a disulfide-crosslinked low molecular linear polyethyleneimine (CLPEI), and sodium hyaluronate was proved to enhance the transfection efficiency significantly (Xu et al., 2009).

The 4F/4R fragment of TCoV S protein encompassing amino acid residue 482–678 has been shown to contain neutralizing epitopes from a previous study (Chen et al., 2011). To determine whether fragment of TCoV S protein could stimulate protective neutralizing antibody response, DNA vaccines encoding 4F/4R S fragment gene (DNA-4F/4R), the ternary complex composed of DNA-4F/4R, CLPEI, and HA (DPH-4F/4R), and recombinant 4F/4R S fragment protein were generated. Thus, the purpose of the present study was to determine the immune response and protection efficacy of DNA vaccination against infectious challenge in a prime-boost strategy by priming with naked DNA-4F/4R or DNA in PEI nanoparticle DPH-4F/4R, and boosting with recombinant 4F/4R S fragment protein.

2. Materials and methods

2.1. Virus

Turkey coronavirus Indiana isolate 540 (TCoV 540) was propagated and titrated in 22-day-old embryonated turkey eggs as previously described (Loa et al., 2001). Briefly, intestines harvested from embryonated eggs inoculated with TCoV 540 were homogenized and served as virus stock for further titration and challenge in turkeys. To determine the median embryo infectious dose (EID_{50}) of virus stock TCoV 540, 10-fold serially diluted virus stock from 10^{-3} to 10^{-7} were inoculated into five 22-day-old embryonated turkey eggs per dilution. After 3 days of inoculation, the embryonic intestines were harvested and subjected to immunofluorescent antibody (IFA) assay with antiserum to TCoV 540 to determine the dose that infected 50% of embryos by the method of Reed and Muench (Thayer and Beard, 2008).

2.2. Eggs and poults

Turkey eggs and one-day-old turkey poults were obtained from Perdue Farm (Thorntown, Indiana, USA).

Turkey eggs were incubated in an incubator at 37.5 °C with a humidity level of 55% (Jamesway, Indian Trail, NC, USA) and turkey poults were housed in isolated floor pens. Feed and water were provided *ad libitum*. Sera collected from one-day-old turkey poults were negative for TCoV-specific Ab. The protocol for care and use of turkey eggs and turkey poults in the present study was approved by the Purdue University Animal Care and Use Committee.

2.3. Construction of DNA vaccine encoding TCoV spike protein 4F/4R fragment

The gene encoding amino acid residues 482–678 (4F/4R) of the S protein of TCoV 540 (GenBank accession number EU022525) amplified by primer 4F, 5′-GGCCATGGCGTGCCAGACAGT-3′, and 4R, 5′-CAAAGGTACCCAAATTAGATACAT-3′ was cloned into the *Nco*I and *Kpn*I sites of expression vector pTriEx3 (Novagen, Madison, WI, USA) in frame and upstream of the series of Histidine residues as construct pTriEx3-4F/4R. Plasmid pTriEx3-4F/4R was purified by QIAfilter™ plasmid Giga kit (Qiagen, Valencia, CA, USA) from the lysogeny broth (LB) culture of *Escherichia coli* (*E. coli*) strain DH5α transformed with pTriEx3-4F/4R. The expression of TCoV 4F/4R S fragment from the purified plasmid pTriEx3-4F/4R was verified by IFA with turkey anti-TCoV 540 serum on the COS-7 cells transfected with pTriEx3-4F/4R by using FuGENE® HD transfection reagent (Roche, Indianapolis, IN, USA) in the ratio of 3:2 in reagent (μl) to DNA (μg) according to the procedures described in a previous study (Chen et al., 2011).

2.4. Formation of DNA-CLPEI-HA (DPH) ternary complex

Disulfide-crosslinked polyethyleneimine was provided by Dr. Yoon Yeo and formation of DNA-CLPEI-HA (DPH) complex was prepared by Dr. Peisheng Xu. Solutions of DNA pTriEx3-4F/4R (0.05 μg/μl) and CLPEI (0.5 μg/μl) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered saline (HEPES, 10 mM, pH 7.2) were mixed (v/v, 1:1) and incubated at room temperature for 30 min to prepare a complex of DNA and CLPEI (DP) first. Sequentially, the DP complex was added to HA solution in HEPES buffered saline with a HA/DNA ratio (w/w) of 1 and incubated at room temperature for 10 min. The DPH complex was then frozen in liquid nitrogen and lyophilized completely. Before inoculation, the powder of DPH was reconstituted with sterile water to the concentration of 0.5 μg/μl for intramuscular injection. The expression of TCoV 4F/4R S fragment from the purified plasmid pTriEx3-4F/4R was verified by IFA with turkey anti-TCoV 540 serum on the COS-7 cells transfected with 4 μg of DPH complex consisting of pTriEx3-4F/4R without transfection reagent.

2.5. Production of recombinant TCoV S protein 4F/4R fragment from E. coli

The construct pTriEx3-4F/4R was transformed into *E. coli* strain Rosetta and the expressed TCoV 4F/4R S fragment was harvested and purified according to the protocol described previously (Chen et al., 2011). The size (26 kDa) and purity of the produced TCoV 4F/4R S fragment were confirmed by SDS-PAGE and Western blotting analysis.

2.6. Vaccination and challenge

One-day-old turkey poults were used in two trials of vaccination and challenge experiments. The vaccination schedule is summarized in Table 1. Two vaccinations (2V) in trial 1 and three vaccinations (3V) in trial 2 were scheduled and followed by viral challenge. There were 15 turkeys per group and every 5 turkeys in each group were sacrificed at one day before challenge, 3 days post viral inoculation (dpi), and 10 dpi in both trials. Group NC-2V in trial 1 and group NC-3V in trial 2 serving as negative control that received PBS instead of DNA vaccine, protein, or virus. The turkeys in group VC-2V in trial 1 and group VC-3V in trial 2 were injected intramuscularly with DNA plasmid vector pTriEx3 or PBS with complete Freud's adjuvant (CFA) (Sigma–Aldrich, St. Louis, MO, USA) as the vector control groups. These two groups became challenge control groups after oral challenge by 100 EID_{50} of TCoV 540, respectively. In trial 1, turkeys in G1-750DP were primed with one dose of 750 μg pTriEx3-4F/4R on day 1 and boosted with 200 μg of TCoV 4F/4R S protein with CFA on day 14 before viral challenge on day 28. In trial 2, DNA vaccine pTriEx3-4F/4R was given on day 1 and 7, TCoV 4F/4R S protein in CFA on day 21, and viral challenge on day 35. The turkeys in G2-100DPH received one dose of 100 μg pTriEx3-4F/4R in the DPH complex consisting of DNA, polymer CLPEI, and HA, one dose of 750 μg pTriEx3-4F/4R, and one dose of 200 μg TCoV 4F/4R S protein with CFA before viral challenge. The turkeys in G3-750DDP were vaccinated intramuscularly with two doses of 750 μg pTriEx3-4F/4R and one dose of 200 μg TCoV 4F/4R S protein with CFA before viral challenge.

2.7. Clinical signs and necropsies

In both trials, clinical signs including depression, diarrhea, and ruffled feathers were monitored for 10 days after viral challenge. Turkey poults weighted to evaluate the status of decreased body weight gains caused by TCoV infection. Sera were collected before challenge, at 3 and 10 dpi to measure TCoV-specific antibody response by performing TCoV 4F/4R S fragment-based ELISA and virus neutralization (VN) assay. At one day before challenge, 3 and 10 dpi, turkey poults from all groups were necropsied and various tissue, including thymus, spleen, ileum, jejunum, bursa, and cecal tonsil, were collected for histopathological examination. The ileum and jejunum samples were subjected to IFA assay for detecting TCoV antigen. The ileum samples were used to determine TCoV viral RNA load by quantitative real-time RT-PCR (qRT-PCR). The spleen samples were processed to determine turkey IFNγ mRNA level by qRT-PCR.

2.8. IFA for detecting TCoV antigen

A 5-cm long segment of jejunum adjacent to the Merckel's diverticulum and a 5-cm long segment of ileum between the paired ceca were collected. Contents in the

Table 1
Vaccination schedule of TCoV 4F/4R spike protein fragment-based DNA vaccination in DNA-prime protein-boost regimen.

Trial 1				
Age	1-day-old		14-day-old	28-day-old
Groups[a]	DNA prime		Protein boost	Challenge
NC-2V	PBS		PBS	PBS
VC-2V	750 μg pTriEx3		PBS w/CFA[b]	$100EID_{50}$[c]
G1-750DP	750 μg pTriEx3-4F/4R		200 μg 4F/4R Pro w/CFA	$100EID_{50}$
Trial 2				
Age	1-day-old	7-day-old	21-day-old	35-day-old
Groups	1st DNA prime	2nd DNA prime	Protein boost	Challenge
NC-3V	PBS	PBS	PBS	PBS
VC-3V	750 μg pTriEx3	750 μg pTriEx3	PBS w/CFA	$100EID_{50}$
G2-100DPH	100 μg pTriEx3-4F/4R-DPH	750 μg pTriEx3-4F/4R	200 μg 4F/4R Pro w/CFA	$100EID_{50}$
G3-750DDP	750 μg pTriEx3-4F/4R	750 μg pTriEx3-4F/4R	200 μg 4F/4R Pro w/CFA	$100EID_{50}$

[a] Groups: in trial 1, turkeys received two doses of vaccines (2V): NC-2V, negative control turkeys; VC-2V, turkeys received one dose of 750 μg vector pTriEx3 and PBS; G1-750DP, turkeys inoculated with one dose of 750 μg pTriEx3-4F/4R and one dose of 200 μg TCoV 4F/4R spike protein (4F/4R Pro). In trial 2, three doses of vaccines were given (3V): NC-3V, negative control turkeys; VC-3V, turkeys received two doses of 750 μg vector pTriEx3 and PBS; G2-100DPH, turkeys primed with one dose of 100 μg pTriEx-4F/4R in DPH-complex (a complex of DNA, disulfide-crosslinked polyethyleneimine (CLPEI), and sodium hyaluronate (HA) complex), one dose of 750 μg pTriEx3-4F/4R, and boosted with 200 μg 4F/4R Pro; G3-750DDP, turkeys primed with 2 doses of 750 μg pTriEx3-4F/4R and boosted with 200 μg 4F/4R Pro.
[b] CFA, complete Freud's adjuvant.
[c] EID_{50}, the effective dose infecting 50% of inoculated embryos.

intestinal segments collected were rinsed away by using cold PBS. The intestinal segments were folded and wrapped in a sheet of aluminum foils and then frozen at −80 °C. The frozen specimens were trimmed at −20 °C and three 6-nm sections from the ileum and three from the jejunum per sample were prepared for IFA. The antiserum to TCoV 540 as the primary Ab and FITC-conjugated goat anti-turkey IgG (H + L) Ab (KPL, Gaithersburg, MD, USA) as the secondary Ab were used to stain the acetone-fixed sections of frozen ileum and jejunum samples. Each section was observed under a fluorescent microscope and IFA scores from three independent fields per section were determined. The infectivity of TCoV in turkeys was determined by the sum of IFA scores presented in three categories modified from a previous study by our laboratory (Loa et al., 2001): (1) strength of positive signals scaled from negative (0), weak (1), moderate (2), to strong (3); (2) distribution of positive cells ranged from negative (0), focal (1), multifocal (2), to diffuse (3); (3) number of positive cells can be negative (0), low (1), medium (2), or high (3).

2.9. ELISA and VN assay

The procedures of TCoV 4F/4R S fragment-based ELISA and VN assay were described previously (Chen et al., 2011). In brief, the serum samples were diluted at 1:200 and reacted to the TCoV 4F/4R S fragment coated on the plate. Bound antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-turkey IgG Ab diluted at 1:40,000 and the optical density at 450 nm (OD 450 nm) was read with VMax™ ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). For VN assay, sera were heat-inactivated at 56 °C for 30 min and then fourfold diluted serially. The diluted serum was incubated with 20 EID_{50} of TCoV 540 at 37 °C for 1 h and the mixture was subsequently inoculated into five 22-day-old turkey embryonated eggs *via* amniotic sac route per dilution of each serum sample. The intestines of turkey embryos were harvested after incubation for three days and examined by IFA using TCoV 540 antiserum. The dilution of serum preventing the infection of TCoV 540 in 50% of inoculated eggs was calculated as VN titer by Reed and Muench method (Thayer and Beard, 2008).

2.10. qRT-PCR for turkey IFNγ mRNA level

Total RNA was extracted from spleens in RNA*later*® stabilization reagent (Qiagen, Valencia, CA, USA) by using RNeasy mini kit (Qiagen, Valencia, CA, USA). The extracted RNA was treated with RNase-free DNase (Qiagen, Valencia, CA, USA) to remove possible genomic DNA contamination. Single stranded cDNA was synthesized using random primer and SuperScript III reverse transcriptase (Invitrogen, San Diego, CA, USA). The cDNA was subjected to SYBR Green-based qRT-PCR with forward primer TKIFNGF, 5′-GTGAAGAAGGTGAAAGATATCATGGA-3′, and reverse primer TKIFNGR, 5′-ACTTTGCGCTGGATTCTCA-3′ specific to turkey IFNγ mRNA. The reaction with GADPH forward primer, 5′-ATCAAGAGGGTAGTGAAGGCTGCT-3′, and GADPH reverse primer, 5′-TCAAAGGTGGAGG AATGGCTGTCA-3′, targeting GADPH mRNA was used as internal control for SYBR Green-based qRT-PCR. The SYBR Green-based qRT-PCR mixture containing 200 nM forward and reverse primers and 1× EXPRESS SYBR Green qPCR universal qPCR Supermixes (Invitrogen, Carlsbad, CA, USA) were used. The parameters for thermal cycling were 2 min at 50 °C, 2 min at 95 °C, 40 cycles of 95 °C for 20 s with acquiring SYBR Green signal at 60 °C for 60 s, and 5 min at 60 °C in a real-time PCR cycler (Rotor-Q, Qiagen). Melting curve analysis was performed from 65 °C to 95 °C, with a ramp speed of 1 °C/s. The result of the melting curve analysis was used to evaluate the specificity of the amplification product. The cycle threshold (Ct) value of IFNγ gene was normalized with the Ct value of GADPH. The mRNA level

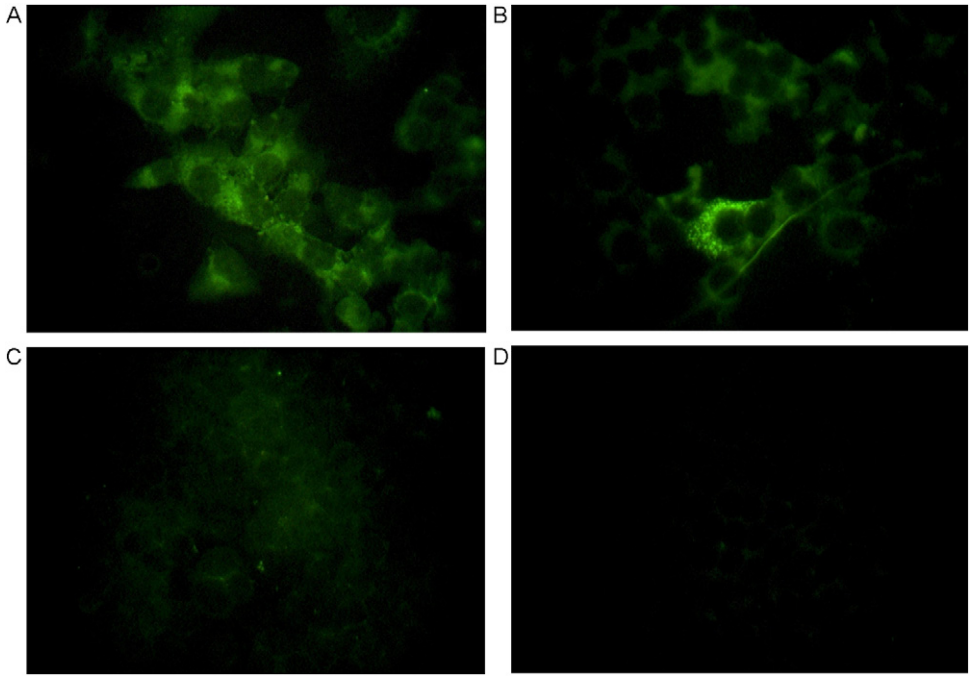

Fig. 1. Expression of encoded TCoV 4F/4R spike protein fragment (482–678) from plasmid pTriEx3-4F/4R in COS-7 was detected by immunofluorescent antibody assay (IFA) 72 h after transfection. (A) COS-7 cells transfected with pTriEx3-4F/4R using FuGEGE® HD transfection reagent (Roche) show positive signals to turkey anti-TCoV serum, 400×; (B) COS-7 cells transfected with pTriEx3-4F/4R in DNA-CLIPEI-HA (DPH) complex show positive signals to turkey anti-TCoV serum, 400×; (C) COS-7 cells transfected with vector pTriEx3 using FuGEGE® HD transfection reagent have negative immunofluorescence to turkey anti-TCoV serum, 400×; and (D) COS-7 cells without transfection have negative immunofluorescence to turkey anti-TCoV serum, 400×.

of IFNγ in group VC-2V and Group 1-750DP was compared to that in group NC-2V while the IFNγ mRNA level in group VC-3V, Group 2-100DPH, and Group 3-750DDP was compared to that in group NC-3V. The fold change of IFNγ gene expression was calculated as $2^{-\Delta\Delta Ct}$ where $\Delta Ct_{IFN\gamma} = Ct_{IFN\gamma} - Ct_{GADPH}$ and $\Delta\Delta Ct_{IFN\gamma}$ = each $\Delta Ct_{IFN\gamma}$ in each group – average of $\Delta Ct_{IFN\gamma}$ in corresponding negative control group.

2.11. qRT-PCR for TCoV viral RNA load

With modifications from a previous study (Chen et al., 2010), the cDNA reverse transcribed from total RNA of collected ileum samples in RNA*later*® stabilization reagent was subjected to TaqMan probe-based qRT-PCR with primers QS1F and QS1R, and probe QS1P specific to TCoV S2 gene. The cycling profile was 5 min of 94 °C followed by 40 cycles of 94 °C for 20 s and 61 °C for 1 min with acquiring the fluorescence FAM in a real-time PCR cycler (Rotor-Q, Qiagen). The concentration of TCoV (fg/μl) was calculated by absolute quantitative standard curve developed by 10^{-3} to 10^{-8} of 10-fold diluted plasmid pTriEx3-6F/6R (nucleotide position 2490–3213 from start codon of S gene from TCoV 540) with known concentration measured by GeneQuant pro RNA/DNA calculator (GE Healthcare Bioscience, Piscataway, NJ, USA). When the Ct value of the sample was larger than the Ct value of non-template control (NTC) or larger than the total cycle number of 40 or the signal was below threshold, the concentration of TCoV in the sample was considered as 0 fg/μl.

2.12. Statistical analysis

The normality of the data was evaluated by quantile–quantile (QQ) plots. When the data did not distribute normally, nonparametric Mann–Whitney *U* test was used to compare the data from two groups and Kruskal–Wallis *H* test was used to analyze the data from three and more groups. The normally distributed data underwent independent *t*-test or one-way analysis of variance (ANOVA). All statistical analyses used SPSS 17.0 program (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Expression of plasmid pTriEx3-4F/4R in COS-7 cells

The green fluorescence was seen in the cytoplasm of the COS-7 cells transfected with pTriEx3-4F/4R with transfusion reagent or DPH complex without transfusion reagent, indicating the expression of TCoV 4F/4R S fragment in COS-7 cells (Fig. 1). Purified TCoV 4F/4R S fragment was detected by the serum collected from TCoV-infected turkey on Western blotting with a size of 26 kDa (Fig. 2).

3.2. Protection efficacy against challenge by TCoV

In both trials, the body weights of turkeys from each group had no significant differences ($p > 0.05$) before viral challenge (Table 2). After challenge by TCoV, the turkeys

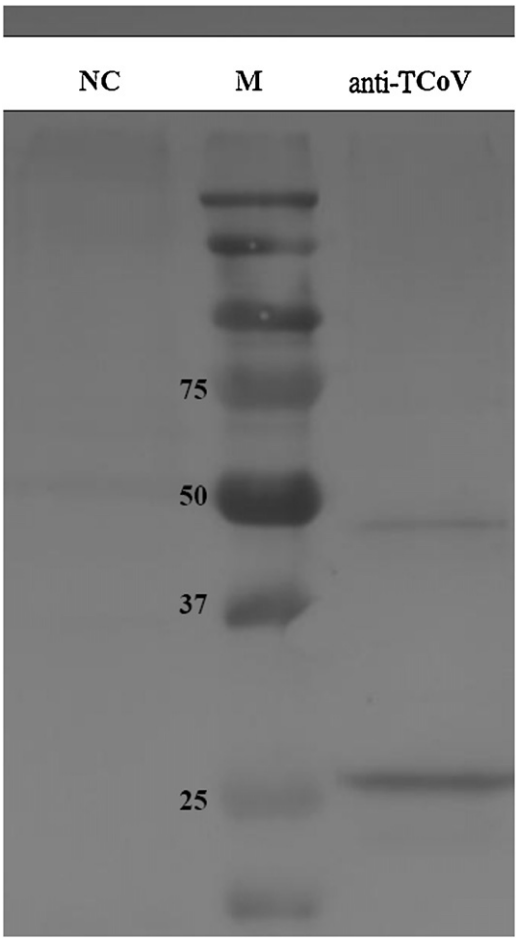

Fig. 2. Western blotting of TCoV 4F/4R spike protein fragment (482–678) reacted to the serum collected from TCoV-infected turkeys. Lane "NC" is TCoV 4F/4R S protein reacted to the serum from non-infected turkey. Lane "M" is protein marker in kDa. Lane "anti-TCoV" is TCoV 4F/4R S protein reacted to the serum from TCoV-infected turkey. The darker band at 26 kDa is monomer of TCoV 4F/4R S protein and the lighter band below 50 kDa is dimer of TCoV 4F/4R S protein.

in G1-750DP weighed significantly heavier than those in group VC-2V ($p < 0.05$) but lighter than NC-2V turkeys ($p < 0.05$) at 3 dpi in trial 1. In trial 2, the vaccinated turkeys in G2-100DPH and G3-750DDP were heavier than the non-vaccinated turkeys in groups VC-3V and NC at 3 dpi, however, there were no significant differences ($p > 0.05$).

DNA vaccination with a DNA-prime protein-boost approach targeting TCoV 4F/4R S fragment containing neutralizing epitopes reduced the number of turkeys showing clinical signs, decreased the intensity, number and distribution of IFA-positive enterocytes and diminished the viral load in the ileum of TCoV-infected turkeys (Table 3). In trial 1, the turkeys in G1-750DP showed less clinical signs and significantly lower IFA scores ($p < 0.05$) than the un-vaccinated turkeys at 3 and 10 dpi. The viral RNA loads determined by qRT-PCR were also lower in the ileum of vaccinated turkeys than un-vaccinated turkeys although there was no statistical difference ($p > 0.05$) probably due to sample variations. In trial 2, the IFA scores and viral loads

Table 2
The body weights of vaccinated turkeys and turkeys in negative and vector control groups before challenge, 3 and 10 days post inoculation (dpi) of turkey coronavirus.

Groups[a]	Pre-challenge	3 dpi	10 dpi
Trial 1			
NC-2V	1028 ± 112[b]	1460 ± 124[e]	2015 ± 290[b]
VC-2V (CC)[a]	949 ± 126[b]	1000 ± 146[c]	1678 ± 164[b]
G1-750DP	999 ± 133[b]	1251 ± 26[d]	1952 ± 163[b]
Trial 2			
NC-3V	1685 ± 91[b]	2015 ± 290[b]	2992 ± 214[b]
VC-3V (CC)[a]	1502 ± 307[b]	1883 ± 184[b]	2964 ± 228[b]
G2-100DPH	1507 ± 410[b]	2089 ± 111[b]	2409 ± 589[b]
G3-750DDP	1789 ± 129[b]	2021 ± 151[b]	2414 ± 408[b]

[a] Groups: same as the detailed description in Table 1. Group VC-2V and VC-3V become challenge control (CC) groups after the challenge of turkey coronavirus.
[b] No significant difference ($p > 0.05$) is shown among the groups at the same time point in the same trial by ANOVA Tukey HSD analysis.
[c] At 3 dpi in Trial 1, three groups labeling with different letter above the average with standard deviation have significant difference by ANOVA Tukey HSD analysis ($p < 0.05$).
[d] At 3 dpi in Trial 1, three groups labeling with different letter above the average with standard deviation have significant difference by ANOVA Tukey HSD analysis ($p < 0.05$).
[e] At 3 dpi in Trial 1, three groups labeling with different letter above the average with standard deviation have significant difference by ANOVA Tukey HSD analysis ($p < 0.05$).

were lower in turkeys from G2-100DPH and G3-750DDP than those in non-vaccinated turkeys at 3 and 10 dpi. The turkeys in G2-100DPH had lower viral RNA loads in the ileum than those in G3-750DDP and un-vaccinated turkeys. Turkeys in G2-100DPH also had significantly lower IFA scores than those in the other groups at 10 dpi ($p < 0.05$).

3.3. Antibody responses to vaccination by ELISA

Vaccination of TCoV 4F/4R S fragment containing neutralizing epitopes induced significant antibody response in both trials (Fig. 3). At one day before viral challenge, 5 out of 15 turkeys in G1-750DP, 6 out of 15 turkeys in G2-100DPH, and 11 out of 15 turkeys in G3-750DDP had the levels of antibodies over O.D. value of 0.5. Significantly increased antibody levels ($p < 0.05$) were detected in the turkeys in G1-750DP at 3 dpi compared to those in G1-750DP before challenge in trail 1. In trial 2, the levels of antibodies in the turkeys in G3-750DDP were higher than those in G2-100DPH before and after challenge with no statistical significance ($p > 0.05$). The levels of antibodies in turkeys in G2-100DPH and G3-750DDP were significantly higher ($p < 0.05$) than those found in un-vaccinated turkeys before and after challenge. With one more dose of 750 μg DNA vaccine pTriEx3-4F/4R, the turkeys in G3-750DDP produced significantly higher ($p < 0.05$) antibodies than those in G1-750DP before viral challenge. With an extra dose of 100 μg DNA vaccine incorporated with PEI and HA, the turkeys in G2-100DPH elicited higher antibodies than those in G1-750DP before challenge although no significant differences were found ($p > 0.05$). In both trials, vaccinated turkeys responded to TCoV infection much faster than non-vaccinated turkeys and had significantly increased antibody levels detected at 3 dpi ($p < 0.05$).

Table 3
Protection efficacy of DNA vaccine targeting TCoV 4F/4R spike fragment containing neutralizing epitopes at 3 and 10 days post inoculation (dpi) of turkey coronavirus.

	Groups[a]	Clinical signs[b]	IFA (positive/total)	IFA score[c]	qRT-PCR[d] (fg/μl)
Trial 1					
3 dpi	NC-2V	0/5	0/5	0[e]	0
	VC-2V (CC)	5/5	5/5	8.2 ± 0.8[g]	35–1.565 (484 ± 631)
	G1-750DP	1/5	4/5	5.1 ± 1.4[f]	12–206 (124 ± 80)
10 dpi	NC-2V	0/5	0/5	0[f]	0
	VC-2V (CC)	5/5	5/5	4.8 ± 1.1[e]	42–584 (201 ± 221)
	G1-750DP	1/5	3/5	2.1 ± 2.1[f]	4–273 (63 ± 117)
Trial 2					
3 dpi	NC-3V	0/5	0/5	0[e]	0
	VC-3V (CC)	5/5	5/5	8.2 ± 1.3[f]	431–3413 (1392 ± 1303)
	G2-100DPH	2/5	3/5	3.5 ± 3.5[e]	0–410 (108 ± 171)
	G3-750DDP	2/5	3/5	3.5 ± 3.7[e]	4–619 (258 ± 273)
10 dpi	NC-3V	0/5	0/5	0[e]	0
	VC-3V (CC)	5/5	5/5	4.6 ± 0.9[f]	24–1015 (305 ± 432)
	G2-100DPH	0/5	3/5	1.5 ± 1.7[e]	4–42 (18 ± 16)
	G3-750DDP	2/5	3/5	2.3 ± 2.6[e,f]	4–223 (102 ± 105)

[a] Groups: same as the detailed description in Table 1. Group VC-2V and VC-3V become challenge control (CC) groups after the challenge of turkey coronavirus.
[b] Number of turkeys showing clinical signs, including diarrhea and distended intestines is presented out of total 5 turkeys in each group at each time point.
[c] IFA score is the sum of scores assigned by three categories of IFA responses: (1) strength of positive signals: negative (0), weak (1), moderate (2), strong (3); (2) distribution of positive cells: negative (0), focal (1), multi-focal (2), diffuse (2); (3) number of positive cells: negative (0), low (1), medium (2), high (3).
[d] qRT-PCR is quantitative real-time RT-PCR used to measure the viral load (TCoV S2 gene) in the intestines samples, presented in the range of TCoV concentration (fg/μl) followed by average with standard deviation detected from 5 turkeys in each group at each time point. No significant differences were found between groups by Kruskal–Wallis H analysis ($p > 0.05$).
[e] The different letters indicate significant difference among groups by ANOVA Tukey HSD analysis ($p < 0.05$).
[f] The different letters indicate significant difference among groups by ANOVA Tukey HSD analysis ($p < 0.05$).
[g] The different letters indicate significant difference among groups by ANOVA Tukey HSD analysis ($p < 0.05$).

3.4. VN antibody responses to vaccination

Significantly higher ($p < 0.05$) VN antibodies against TCoV were elicited in vaccinated turkeys before and after challenge by TCoV (Fig. 4). In both trials, the serum collected from vector control turkeys before viral challenge could not neutralize TCoV infection in embryonated eggs and the VN titers in the serum collected from non-vaccinated turkeys at 3 and 10 dpi were from 4 to 8. At one day before viral challenge, the VN titers over 4 were detected in 2 out of 5 turkeys in G1-750DP, 1 out of 5 turkeys in G2-100DPH, and 2 out of 5 turkeys in G3-750DDP. At 3 dpi, 4 out of 5 turkeys in G1-750DP, 2 out 5 turkeys in G2-100DPH, and 5 out of 5 turkeys in G 3-750DDP produced the VN titers over 4. At 10 dpi, all vaccinated turkeys responded to the infection of TCoV. In trial 1, the VN titer was detectable in turkeys from G1-750DP before challenge, increased significantly at 3 dpi ($p < 0.05$), and declined at 10 dpi. In trial 2, the turkeys in G3-750DDP had higher VN titer than those in G2-100DPH and non-vaccinated turkeys before challenge.

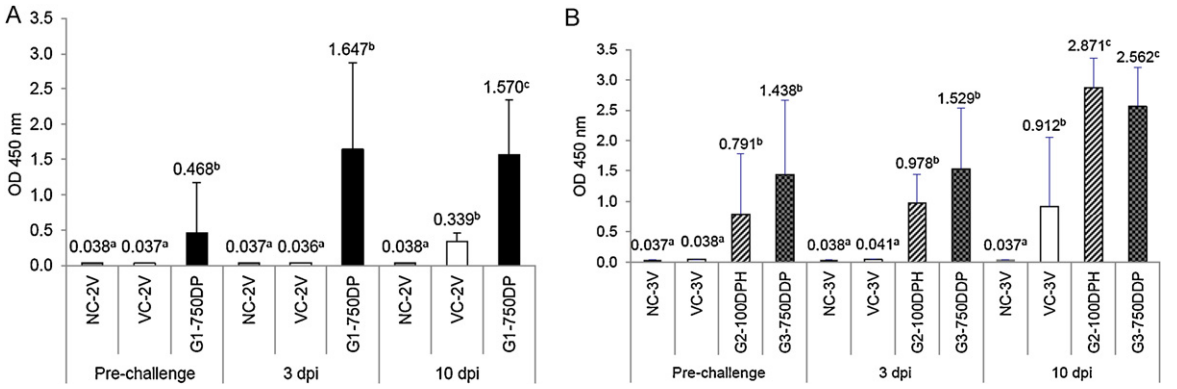

Fig. 3. Serum antibody levels determined by TCoV spike protein-based ELISA (O.D.450 nm) in trial 1 (A) and trial 2 (B). The gray bars are for turkeys in the negative control groups (NC-2V and NC-3V). The white empty bars are for turkeys in the vector control groups (VC-2V and VC-3V). The black bars are from turkeys primed with one dose of 750 μg pTriEx3-4F/4R (S-DNA) and boosted with one dose of 200 μg TCoV 4F/4R S fragment (482–678) (S-Pro) (G1-750DP). The bars with black and white strips are from turkeys primed with one dose of 100 μg pTriEx-4F/4R in DPH-complex, one dose of 750 μg S-DNA, and boosted with S-Pro (G2-100DPH). The bars with checker broad are from turkeys primed with 2 doses of 750 μg S-DNA and boosted with S-Pro (G3-750DDP). The different letters above each bar at each time point indicate significant differences ($p < 0.05$).

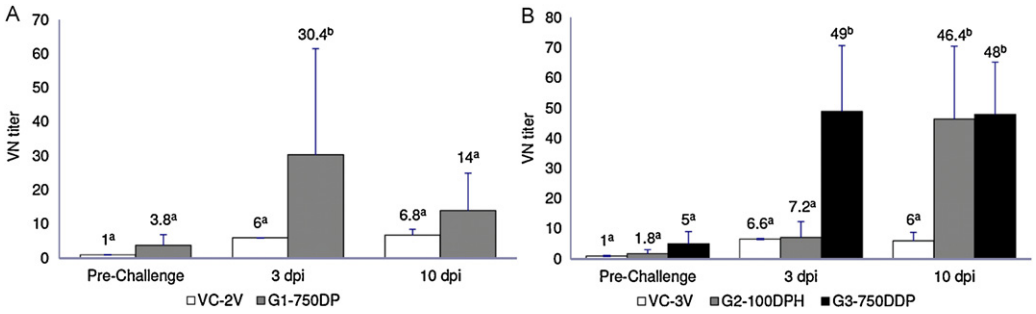

Fig. 4. Serum virus neutralization (VN) titers against TCoV 540 determined by VN assay using 22-day-old turkey embryonated eggs in trial 1 (A) and trial 2 (B). In trial 1, turkeys received two doses of vaccines: NC-2V, negative control turkeys; VC-2V, turkeys received one dose of 750 μg plasmid vector pTriEx3 and PBS; G1-750DP, turkeys primed with one dose of 750 μg pTriEx3-4F/4R (S-DNA) and boosted with one dose of 200 μg TCoV 4F/4R S fragment (482–678) (S-Pro). In trial 2, three doses of vaccines were given: NC-3V, negative control turkeys; VC-3V, turkeys received two doses of 750 μg plasmid vector pTriEx3 and PBS; G2-100DPH, turkeys primed with one dose of 100 μg pTriEx-4F/4R in DPH-complex and one dose of 750 μg S-DNA and boosted with S-Pro; G3-750DDP, turkeys primed with 2 doses of 750 μg S-DNA and boosted with S-Pro. The different letters above each bar at each time point indicate significant differences ($p < 0.05$).

The turkeys in G3-750DDP had faster neutralizing antibody responses than those in G2-100DPH and VC-3V by producing significantly higher level ($p < 0.05$) of neutralizing antibody at 3 dpi while the turkeys in G2-100DPH did not produce significantly higher level ($p > 0.05$) of neutralizing antibody until 10 dpi.

3.5. Turkey IFNγ transcription level

In trial 1, the transcription of IFNγ gene in turkeys from G1-750DP was up-regulated 2.4-fold before viral challenge, 2.06-fold at 3 dpi, and 0.93-fold at 10 dpi (Fig. 5A). Vector control turkeys had lower IFNγ mRNA levels compared to turkeys in G1-750DP before challenge but showed a similar pattern of IFNγ induction after viral challenge. There was no statistical significance ($p > 0.05$) among the different concentrations of IFNγ produced by turkeys from the groups NC-2V, VC-2C, and G1-750DP before and after challenge. In trial 2, the transcription of IFNγ gene in turkeys from VC-3V, G2-100DPH, and G3-750DDP was down-regulated significantly before challenge ($p < 0.05$) and the mRNA levels were only 41, 8, and 18% to the level found in NC-3V turkeys, respectively (Fig. 5B). After challenge, the IFNγ levels increased significantly to 2.4-fold in turkeys from G3-750DDP and VC-3V ($p < 0.05$). Turkeys from G2-100DPH had higher mRNA level of IFNγ at 3 dpi compared to the level detected before challenge. At 10 dpi, all turkeys including NC-3V turkeys had similar transcription level of IFNγ mRNA.

4. Discussion

The present study has demonstrated that the neutralizing epitope-containing TCoV S fragment encompassing the carboxyl-terminus S1 and the amino-terminus S2 (amino acid residue 482–678, 4F/4R fragment) can induce neutralizing antibodies and provide partial protection against infectious challenge by TCoV. The full-length S protein of SARS-CoV has been shown to activate cell-mediated immune responses and induce antibody production to block viral binding, fusion, and infection (He et al., 2006; Kam et al., 2007). Most conformation-dependent neutralizing epitopes are located in the S1 subunit of IBV (Kant et al., 1992) but there are conformation-independent neutralizing epitopes found in 47 amino acids of S1 carboxyl-terminus and 20 amino acids of S2 amino-terminus (Kusters et al., 1989). Chickens inoculated with peptides with antigenic epitopes corresponding to amino

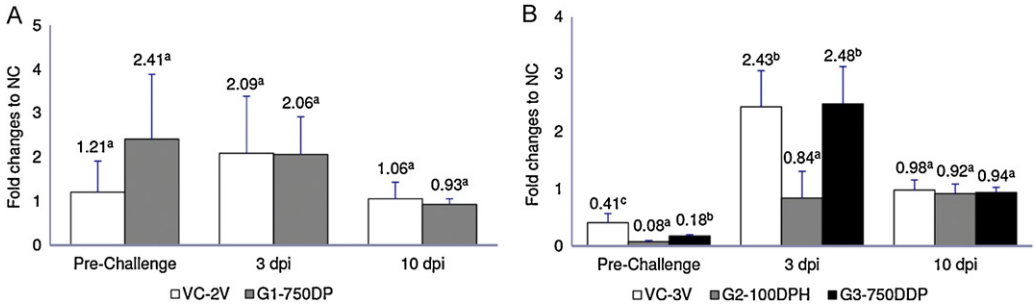

Fig. 5. Fold changes of turkey interferon gamma (IFNγ) mRNA levels in the spleens of turkeys in the vector control and vaccinated groups before challenge and at 3 and 10 days after challenge (dpi) in 2 trials. In trial 1 (A), turkeys in group VC-2V received one dose of 750 μg plasmid vector pTriEx3 and PBS and turkeys in G1-750DP primed with one dose of 750 μg pTriEx3-4F/4R (S-DNA) and boosted with one dose of 200 μg TCoV 4F/4R spike fragment (482–678) (S-Pro). In trial 2 (B), turkeys in group VC-3V received two doses of 750 μg plasmid vector pTriEx3 and PBS, turkeys in G2-100DPH primed with one dose of 100 μg pTriEx-4F/4R in DPH-complex and one dose of 750 μg S-DNA and boosted with S-Pro, and turkeys in G3-750DDP primed with 2 doses of 750 μg S-DNA and boosted with S-Pro. The different letters above each bar at each time point indicate significant differences ($p < 0.05$).

acid residue 294–314 (S1), 532–537 (S1), or 566–584 (S2) of IBV produced antibodies that provided more than 50% protection against IBV infection in kidney but no protection in trachea (Ignjatovic and Sapats, 2005). An exposed domain in the S1 subunit of SARS-CoV from amino acid residue 485–625 induced antibody production to inhibit viral infection (Zhou et al., 2004). These studies suggested that the S fragment of coronavirus contains neutralizing epitopes and is capable of eliciting a protective antiviral antibody response.

DNA vaccination can generate both humoral and cellular immunity specific to protective antigens of various pathogens. After the delivery of DNA plasmids into cells, antigenic proteins are endogenously produced and presented by MHC class I, thus inducing CD8 cytotoxic T-cell responses. Therefore, DNA vaccines have the ability to mimic the effects of live attenuated vaccine without reversion to virulence due to recombination or mutations in live attenuated pathogens. Plasmids can be easily produced in bacteria without the risk of improper folding and instability to express recombinant proteins (Laddy and Weiner, 2006; Coban et al., 2008). Prime-boost strategy and the use of nanoparticles as aid in DNA delivery were undertaken to enhance the immunogenicity and protection efficacy of DNA vaccination in the present study. According to Woo's study (Woo et al., 2005), mice primed intramuscularly with S1 DNA vaccine and boosted with recombinant S1 protein produced by *E. coli* was shown to generate the highest neutralizing antibody titer against SARS-CoV infection compared to DNA vaccine or S1 protein alone. On the other hand, recombinant S1 protein expressed by *E. coli* induced high IgG levels but not high neutralizing antibody titer. This may be due to improper conformation and lack of glycosylation from the recombinant S1 protein expressed in a prokaryotic system. It appears that the conformation and post-translational modification of the S1 protein expressed in DNA-transfected cells is adequate to induce neutralizing antibodies. Thus, priming with DNA vaccine can overcome the issues related to conformation and glycosylation encountered in vaccination or immunization with recombinant S1 protein (Woo et al., 2005). In addition, the use of biodegradable polymeric nanoparticles is one of the potential means to deliver DNA. By combining the advantages of CLPEI and HA, the DNA-CLPEI-HA (DPH) system had a significantly higher ($p < 0.05$) transfection efficiency than the other polymer systems (Xu et al., 2009). The prime-boost regimen using 100 μg DNA in DPH complex as the first dose in the present study provided the same protection as the regimen using 750 μg DNA as the first dose at 3 and 10 dpi. The turkeys vaccinated with 100 μg DNA in DPH complex did not show any local tissue responses and lesions and had lower IFA scores and viral RNA loads in the ileum than the turkeys receiving 750 μg DNA as the first dose. However, the toxicity was revealed when the chickens received 300 μg DNA in DPH complex. Subcutaneous edema and hemorrhage in muscle and liver were observed in the inoculated chickens (unpublished data). Although the application of nanoparticles CLIPEI and HA can increase transfection efficiency of DNA vaccine and enhance protective immune responses triggered by DNA vaccine, but it is also critical to use the appropriate dose that does not cause any toxicity or tissue change.

Prior to challenge, stronger TCoV-specific antibody responses by TCoV S protein-based antibody-capture ELISA, higher neutralizing antibody titers, and more efficacious protection against infectious challenge by TCoV were seen in turkeys primed with two doses of 750 μg DNA vaccine than those primed with only one dose of 750 μg DNA vaccine. Such findings indicated that the frequency of DNA vaccination affected the kinetic and magnitude of the protective antibody response. From the study of infectious bursal disease virus (IBDV), chickens vaccinated with one dose of DNA vaccine showed no protection 10 days after viral challenge while chickens that received 2 or 3 doses of DNA vaccine showed 50–100 or 80–100% protection based on IBDV-induced bursal lesion scores (Chang et al., 2002). Additionally, DNA encoding hemagglutinin protein of influenza virus has been shown to increase hemagglutinin-specific antibody production in a dose-responsive manner with single or multiple injections of DNA (Deck et al., 1997; Robinson et al., 1997). Therefore, the protective antibody response against TCoV infection can be enhanced by increasing the dosage and/or frequency of the DNA vaccine.

The mRNA level of turkey IFNγ determined by qRT-PCR was used to evaluate the cell-mediated immune responses to vaccination and infectious challenge by TCoV because IFNγ is primarily produced by active cytotoxic CD8, Th1 CD4 T cells and natural killer cells. No significant up-regulated transcription of IFNγ was noted after vaccination in the present study, indicating that TCoV 4F/4R S fragment containing the neutralizing epitopes was not actively involved in activation or stimulation of cell-mediated immunity but primarily induced strong humoral immune response to TCoV. Down-regulated IFNγ level can result from the reduced number of natural killer cells, macrophages, activated Th1 CD4 T cells, and activated CD8 T cells (Franke et al., 2006) or the increased number of Th2 CD4 T cells (Zheng et al., 2011). Physical strained and intramuscular injections may result in stress leading to immunosuppression and inhibition of IFNγ production (Greenfeld et al., 2007). In addition, PEI may cause apoptosis and reduce the number of IFNγ-producing cells even though PEI may affect all types of immune cells (Fischer et al., 1999). Therefore, reducing or eliminating stress-induced immunosuppression due to vaccination is an important factor to consider in induction of immunity to DNA vaccination.

Protection provided by vaccination against coronavirus infection involved both humoral and/or cellular immune responses (Seo et al., 2000; Marten et al., 2001; Yang et al., 2004; Thirion and Coutelier, 2009). Vaccinated mice protected from SARS-CoV infection produced high level of IgG and neutralizing antibodies with no significant Th1-biased immune responses (Yang et al., 2004; Woo et al., 2005). The protection was mediated by humoral but not a T-cell dependent immune mechanism because depletion of CD4 and CD8 T cells by using specific monoclonal antibodies did not affect vaccine-induced immunity. In addition, adoptive T-cell transfer showed that donor immune T cells were unable to reduce pulmonary viral replication in recipient

animals while passive transfer of purified IgG from immunized mice provided immune protection (Yang et al., 2004). On the other hand, adoptive transfer of IBV primed αβT cells bearing CD8 antigens protected chicks from IBV infection and passive transfer convalescent IBV serum did not protect chickens against respiratory infection although the viral replication was delayed and kidney disease was prevented (Macdonald et al., 1981; Seo et al., 2000). From studies of mouse hepatitis virus (MHV), acute infection was primarily controlled by CD8 T cells but CD4 T cells, B cells, and NK cells contributed to reduce viral replication (Marten et al., 2001; Thirion and Coutelier, 2009). Thus, both humoral and cellular immunities provide optimal protection against coronaviral infection. DNA vaccination based on N and S1 gene provided more protection against TCoV infection in turkeys than DNA vaccination based on N gene alone in a previous study, suggesting that both humoral and cellular immunities are critical for protection against TCoV in turkeys since DNA vaccine encoding N protein of TCoV can induce cellular immune responses but not antibody responses (Ababneh, 2005). Therefore, future studies of DNA vaccination against TCoV infection will include DNA vaccine encoding neutralizing epitopes in S protein and T cells epitopes in N protein that should generate adequate humoral and cellular immune responses and offer optimal protection against TCoV infection. Because oral administration of vaccine is more suitable in field application and oral vaccination can stimulate protective mucosal immune responses, further investigation on oral delivery of TCoV DNA vaccine by fowl adenovirus (Johnson et al., 2003), attenuated Salmonella (Jiao et al., 2011; Woo et al., 2005), biodegradable nanoparticles (Plapied et al., 2010), or other methods is warranted.

5. Conclusion

DNA-prime protein-boost DNA vaccination targeting TCoV S protein fragment containing neutralizing epitopes can provide partial protection against TCoV infection by eliciting protective humoral immune response to lessen or eliminate clinical signs and reduce the viral loads in the ileum from the infected turkeys.

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