Veterinary Immunology and Immunopathology 218 (2019) 109953

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Evaluation of immunogenicity and protective efficacy of a CpG-adjuvanted DNA vaccine against Tembusu virus

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ARTICLE INFO

Keywords:
Tembusu virus
DNA vaccine
PrM/E proteins
Immune protection

ABSTRACT

Tembusu virus (TMUV) is a contagious pathogen of waterfowl including ducks and geese, which causes symptoms of high fever, loss of appetite and reduced egg production. The development of an effective vaccine is important for the prevention and control of the disease. We evaluated a DNA vaccine based on a recombinant pre-membrane (prM) and envelope (E) protein, using CpG oligodeoxynucleotide (ODN) as an adjuvanted, and tested it for protection efficacy. BHK21 cells were transfected with the recombinant plasmid pVAX1-prM/E-CpG, and the antigenicity of the expressed protein was detected using an indirect immunofluorescence assay (IFA) and western blot assay. One-day-old ducklings were intramuscularly injected with 200 μg doses of pVAX1-prM/E-CpG or pVAX1-CpG, or PBS at ten day intervals. The neutralizing antibodies and cell-mediated immune responses elicited by the DNA vaccine were detected using serum neutralization tests (SNTs) and ELISAs. At 20 days old, the ducks were challenged with 10^3EID50 doses of TMUV SD/02 strain and observed for 15 days post challenge. After the second DNA vaccination and during the monitoring period, the levels of TMUV neutralizing antibodies increased in the pVAX1-prM/E-CpG vaccinated ducks. Vaccination with pVAX1-prM/E-CpG resulted in 100.0% protection of the ducks, whereas approximately 40% of ducks vaccinated with pVAX-CpG or PBS manifested clinical symptoms. Expressions of IFN-γ and IL-6 in the pVAX1-prM/E-CpG group were significantly increased ($p < 0.01$) compared with the control groups during the entire experimental period. The results revealed that a vaccine co-expressing prM and E, and using a CpG-ODN motif as an adjuvant, could elicit effective neutralizing antibody titers and provide efficient protection to ducks against TMUV infection.

1. Introduction

Since April 2010, an outbreak of a new disease in eastern China caused layer ducks to exhibit symptoms of high fever, loss of appetite and reduced egg production, and spread to almost all ducks breeding areas of China. The causative pathogen was identified as TMUV, a member of the *Flaviviridae* family and genus *Flavivirus* (Cao et al., 2011; Su et al., 2011; Tang et al., 2012). Epidemiological surveys showed that TMUV caused mortality in 5%–10% of meat-type ducklings, and layer ducks of a 60%–80% reduction in the annual egg production on duck farms (Cao et al., 2011; Su et al., 2011; Yan et al., 2011).

Flaviviruses are enveloped, single-stranded, positive-sense RNA viruses formed of three structural proteins. Studies have showed that TMUV is maintained in an enzootic cycle among arthropod vectors, wildlife birds and poultry. The envelope protein (E) of flaviviruses possesses three domains (DI, DII and DIII) and facilitates cellular attachment and membrane fusion. The pre-membrane (prM) and NS1 proteins of flaviviruses stimulate the production of antibodies in the host that elicit a strong immune response, providing protection against diseases associated with the infecting virus. DNA vaccines have induced successful protective immunity against other flaviviruses, using the prM, E, and NS1 genes of St. Louis (Phillpotts et al., 1996), West Nile (Davis et al., 2001; Minke et al., 2004), and Japanese encephalitis viruses (Konishi et al., 1998, 2000b). One study showed that a recombinant live duck enteritis virus (DEV) expressing TMUV PrM/E proteins provided complete protection against TMUV challenge, whereas rDEV-TE only conferred partial protection(Chen et al., 2014). It has been reported that mosquitoes and birds may be important in the transmission of TMUV (Tang et al., 2015, 2013). Currently, several types of oil-emulsion inactivated TMUV vaccines are used on many duck farms, but none of them provide total protection against TMUV infection (Lin et al., 2015). Our efforts are focused on the development of a recombinant subunit vaccine against TMUV, using the viral prM and E proteins as target antigens.

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https://doi.org/10.1016/j.vetimm.2019.109953
Received 11 January 2019; Received in revised form 9 June 2019; Accepted 26 September 2019

In recent years, CpG-ODN has been accepted as an immune-adjuvant for various vaccines in animals and humans. The adjuvant activity of CpG-ODN facilitates the activation of innate and adaptive immune responses. CpG-ODN also directly activates monocytes, macrophages and dendritic cells to secrete TNF-α, GM-CSF, chemokines and other cytokines, which subsequently stimulate T helper (Th) cells to secrete additional cytokines that activate natural killer (NK) cells and stimulate IFN-γ production (Jakob et al., 1998; Kemp et al., 2003; Zhu and Marshall, 2001).

Herein, a DNA-based vaccine was constructed which induced neutralizing antibodies and protected ducks against TMUV infection. This vaccine candidate was tested for expression of prM-E proteins in BHK21 cells. This was followed by in vitro verification of the recombinant protein expression, the ability to induce an immune response in ducks, and the ability to protect ducks against TMUV. The results indicated that the recombinant plasmid could be a candidate subunit vaccine for effective immunization against TMUV.

2. Materials and methods

2.1. Ethics statement

All procedures involving animals were approved and authorized by the Animal Care and Use Committee of Shandong Agricultural University (Permit number: 20120618). All experimental animals and treatment in this study were performed in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). Ducks were euthanized using CO_2 for 20 min when animals observed severe symptoms, e.g. depression, anorexia, paralysis, or head tremor. Diet, exercise, vigor, and body weight of animals were monitored daily throughout the experiment.

2.2. Animals, virus propagation and cell culture

BHK-21 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, New Zealand) in an incubator at 37 °C with a constant supply of 5% CO_2. Tembusu virus SD/02 strain was originally isolated from a layer duck farm in Shandong and conserved at the Poultry Diseases Institute, Shandong Agricultural University, Tai'an, Shandong province, China. Ducklings were purchased from Yike Ltd., Shandong, China, and monitored for TMUV and maternal antibodies. They were then divided into groups and kept in separated isolators, respectively.

2.3. Construction of recombinant plasmid

Total RNA of TMUV was extracted using Trizol reagent (Invitrogen, USA), then reverse transcribed into cDNA as a template for PrM/E synthesis. Based on the GenBank sequence (accession no. JF270480), a pair of TMUV prM/E genes specific primers were designed by Primer Premier 5.0 to amplify a PCR product of 2, 096 bp in length. The following forward and reverse primers were designed: prM/E-F: 5′-TTGG TACCGAGCTC*GGATCC*AGCCACCATGGTAAAGAGGACGATT-3′, prM/E-R: 5′-CACACTGGACTAGT*GGATCC*GTGTCACCCAAAGCAA-3. The underlined sequence represents a *Kozak* consensus sequence. The following Synthetic CpG-ODN, 5′-*GAATTC*TCGTCGTT -TTGTCGTTTTGT CGTTTCGTCGTTTTGTCGTTTTGTCGTTTCGTCGTTTTGTCGTTTTGTCG TT*TCTAGA*-3′, containing 12 CpG motifs, and *EcoR* I and *Xba* I restriction enzyme sites, was formulated as the molecular adjuvant to boost the immune responses elicited by the vaccine in ducks. The synthetic CpG-ODN was digested using *EcoR* I and *Xba* I restriction enzymes, and cloned into the pVAX1 vector (Invitrogen, USA). The PCR products were purified from agarose gels and cleaved using *BamH* I restriction enzyme, and then sub-cloned into the linearized pVAX1-CpG using a CloneEZ® PCR Cloning Kit (GenScript, China).

2.4. Detection of mRNA and recombinant protein in vitro

The recombinant plasmid pVAX1-prM/E-CpG was isolated and purified using an Endo-free Plasmid Mini Kit I (Omega, USA) and transfected in to BHK21 cells using Lipofectamine™ 2000 (Invitrogen, USA). The mRNA was extracted from BHK-21 cell lysates 48 h after transfection, and semi-nested RT-PCR was used to confirm the expression of PrM/E. The presence of prM/E genes was confirmed via agarose gel electrophoresis. After 72 h of incubation, the transfected BHK-21 cells were harvested and treated with NP-40 cell lysis buffer (Beyotime, China). The protein samples were separated using SDS-PAGE and transferred electrophoretically onto a polyvinylidene difluoride membrane (Roche, Germany). Recombinant PrM/E protein was detected using mouse anti-TMUV serum (1:20 dilution) as the primary antibody, and goat anti-mouse IgG (H + L) (BoAoSeng, China) was used as the secondary antibody. A colorimetric reaction was observed using the 3, 3′-diaminobenzidine (DAB) Enhanced Liquid Substrate System (TianGen, China)

Mouse anti-TMUV serum was used to detect the recombinant prM/E multi-protein expressed in the transfected cells using an indirect immunofluorescence assay (IFA). The transfected cells were cultured in DMEM medium containing 10% FBS (Gibco, New Zealand) for 48 h. The cells were washed three times with PBS and fixed in chilled acetone for 10 min. Mouse anti-TMUV serum diluted in PBS (1:20) was added as the primary antibody, and incubated at 37 °C for 30 min. Goat anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC) (BoAoSeng, China) was used as the secondary antibody (1:2000 dilutions). Finally, the slides were mounted and observed using a fluorescence microscope (Guava, Germany).

2.5. Vaccinantion and virus challenge

One-day-old healthy ducklings were randomly divided into three groups (30 ducks in each group). Ducklings in Group I received a primary (3 dpi) and booster (10 dpi) vaccination with 200 μg of pVAX1-prM/E-CpG; on the same day, Group II received 200 μg of pVAX1-CpG; and Group III received 0.2 mL of PBS, via multi-point leg intramuscular injection. Twenty ducklings served as an additional negative control (Group IV) and were not vaccinated with anything. Ten days post booster vaccination (20 days old), twenty ducks from each group (I, II and III) were housed in a new isolator and intravenously challenged with a 5 × 103 ELD50 dose of TMUV SD/02 strain. Ducks in the negative control group (IV) were not challenged. All of the ducks were monitored for 15 days post challenge. Clinical symptoms (including acute anorexia, ataxia, paralysis, and diarrhea), deaths, and post-mortem changes were recorded. Cloacal swabs were collected daily after challenge and viral shedding was detected using a semi-RT-PCR.

The 10 ducks from each group (I, II and III) that were not challenged and the ducks from Group IV were used for immune response tests. Peripheral blood was drawn from medial metatarsal veins of all the ducks, incubated at 37 °C for 60 min then inactivated at 56 °C for 30 min. Sera samples were prepared from the peripheral blood to measure the titers of neutralizing antibodies at three-day intervals until 24 days post booster vaccination (dpbv). Peripheral blood was centrifuged at 2000 *g* for 20 min through the lymphocyte separation medium (Sigma-Aldrich, Germany) and then washed three times with PBS. The cell pellet was resuspended in PBS and the concentration was adjusted to 5 × 106 cells/mL. Peripheral blood mononuclear cells (PBMCs) were prepared for the detection of CD4+ and CD8 + T Lymphocytes and of IL-6 and IFN-γ mRNA expression levels (described in the following 2 sections).

2.6. Detection of $CD4^+/CD8^+$ T lymphocytes in PBMCs

PBMCs were incubated with mouse anti-duck CD4 or CD8 antibody (Abcam, UK), diluted 1:100 in FACS buffer (PBS containing 1% fetal

calf serum and 0.02% azide), at 4 °C for 60 min. The cells was centrifuged at 2000 *g* for 5 min at 4 °C, washed twice with PBS, and then incubated with goat anti-mouse IgG [1: 100, conjugated to FITC (Sigma-Aldrich, Germany)], at 4 °C for 60 min. Subsequently, cells were washed three times with PBS and resuspended in 0.5 mL PBS. The PBMCs were analyzed using flow cytometry (Guava, Germany) to determine the numbers of CD4 + and CD8 + T lymphocytes.

2.7. Detection of cytokine expressions by real-time RT-PCR

Every three days post challenge, anti-coagulated blood from ducks of each group was drawn from medial metatarsal veins and PBMCs were isolated. Transcript levels of IL-6 and IFN-γ genes, in response to the vaccination, were quantified using quantitative reverse-transcription PCR. Primers and reaction conditions for each gene are described in a previous study (Lee et al., 2010). Quantitative reverse-transcription PCR (qRT-PCR) was carried out on an Applied Biosystems 7500 FAST system (Applied Biosystems, Denmark).

The expressions of IL-6 and IFN-γ genes were analyzed using the relative quantification method described by Pfaffl (Pfaffl, 2001). The qRT-PCR efficiencies were calculated based on the given slopes in the LightCycler software. The corresponding real-time PCR efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/slope]}$. The relative expression ratio of a target gene was computed based on E and the CP deviation of an unknown sample versus a control, and expressed in comparison to a reference gene (GAPDH).

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CP target\,(control-sample)}}{(E_{\text{ref}})^{\Delta CP ref\,(control-sample)}}$$

2.8. Virus neutralization test (VNT)

A virus neutralization test (VNT) was performed to assess the ability of the duck serum to neutralize the Tembusu virus. BHK-21 cells were cultured in 96-well plates. Serial two-fold dilutions of the sera (1:2n) were mixed with an equal volume containing 200 TCID50 of SD/02 strain, and the mixture was incubated for 30 min at 37 °C. The mixtures were then incubated with BHK-21 cells in 96-well plates at 37 °C for additional six-day propagation. The cytopathic effect (CPE) of cells and the number of plaques were monitored. The neutralization titer was taken as the average of three measures and then calculated using the method of Reed and Muench (Haggett and Gunawardena, 1964). Each sample was tested in triplicates.

2.9. Statistical analysis

Data were gathered and analyzed as the mean ± standard deviation (SD) using SPSS 17.0 software. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., USA). One-way analysis of variance (ANOVA) with *t*-test was used to evaluate the significance of the relative expression ratios, and a p-value of < 0.05 was considered to be significant (*), whereas a p-value of < 0.01 was considered to be very significant (**).

3. Results

3.1. Expression of recombinant PrM/E protein

The recombinant plasmid pVAX1-prM/E-CpG was successfully constructed and confirmed using restriction enzyme digestion, PCR, and DNA sequencing (data not shown). The transcription of PrM/E was confirmed using RT-PCR. As expected, a 78.2 kDa band was visible in protein extracts from cells transfected with pVAX1-prM/E-CpG (Fig. 1). Green fluorescence signals were detected in BHK-21 cells transfected with pVAX1-prM/E-CpG (Fig. 2), whereas the cells transfected with pVAX1-CpG, which served as a negative control, did not display any fluorescence. These data indicate that the recombinant PrM/E protein was successfully expressed in vitro.

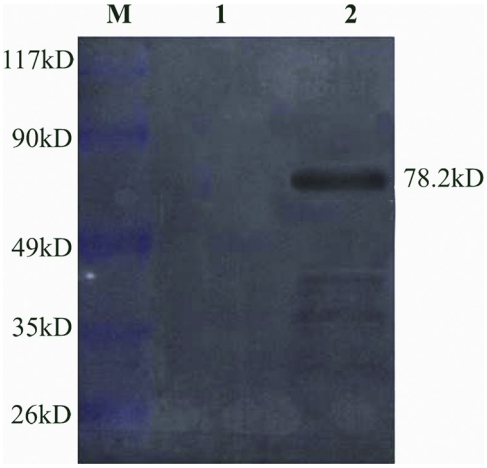

Fig. 1. Detection of PrM/E in BHK21 cells using western blot analysis. Lane M shows a pre-stained protein molecular weight marker (Beyotime, Shanghai, China); Lane 1 shows no translated protein band with pVAX1 alone; Lane 2 shows a 78.2 kDa band, indicating the presence of the translated PrM/E protein.

3.2. Detection of neutralizing antibody

Neutralization titers of all three experimental groups were elevated for at least 24 days post-booster vaccination (dpbv) (ducks were 34 days old). The neutralizing antibody titers against TMUV continuously increased during the 24-day monitoring period. Although the antibodies of Group I (pVAX1-prM/E-CpG) reached a high neutralization titer, those in Groups II and III (pVAX1-CpG and PBS) were quite low, or even undetectable (Fig. 3). These results demonstrate that the subunit DNA vaccine induced specific anti-TMUV neutralizing antibodies in ducks and the booster vaccination elicited a strong antibody response.

3.3. Expression of IL-6 and IFN-γ genes

PBMCs were isolated from ducks at 0, 3, 6, 9, 12, and 15 dpbv, and IL-6 and IFN-γ mRNA expressions were analyzed (Fig. 4). The PBMCs from Group I ducks had significantly up-regulated IL-6 and IFN-γ mRNA expressions ($p < 0.01$) compared with those in Groups II and III (0 dpi). Transcripts of IL-6 and IFN-γ in Group I continuously increased, reaching a peak at 6 dpbv and then decreased. The transcripts were slightly elevated in Group II but remained at baseline in Group III 0–15 dpbv. However, the PMBC expressions of IL-6 and IFN-γ mRNA from Group I was significantly higher than those of Groups II and III at 15 dpbv.

3.4. Changes in $CD4^+/CD8^+$ T lymphocyte ratios

The $CD4^+/CD8^+$ T lymphocyte ratio in peripheral blood was investigated in ducks that received primary and booster vaccinations with different immunological stimulants and those that received no vaccination. The $CD4^+/CD8^+$ T lymphocyte ratios were significantly different among the four groups (Fig. 5). The percentages of $CD4^+$ and $CD8^+$ T lymphocytes from Group I (pVAX1-prM/E-CpG) were significantly higher ($p < 0.01$) than those from Groups II (pVAX1-CpG), III (PBS), and IV (naïve ducks) during the monitoring period, and the percentages of $CD4^+$ and $CD8^+$ T lymphocytes from Group II were significantly higher ($p < 0.05$) than those from Groups III and IV.

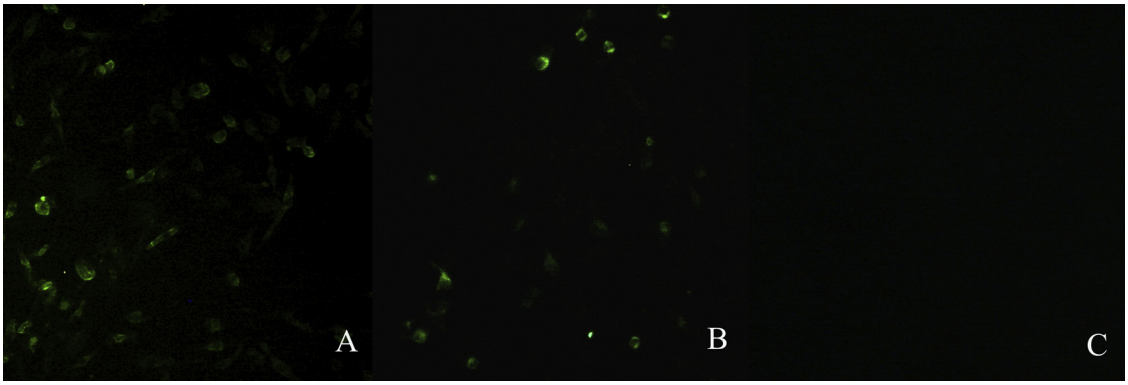

Fig. 2. Expression of pVAX1-prM/E-CpG in BHK-21 cells *in vitro* evaluated using IFA. BHK-21 cells were transfected with 5 μg of pVAX1-prM/E-CpG, incubated at 37 °C for 48 h, and then stained with mouse anti-TMUV serum using an indirect immunofluorescence assay (IFA). The expressed recombinant protein was stained in the cytoplasm. (A) BHK-21 cells transfected with pVAX1-prM/E-CpG; (B) BHK-21 cells infected with Tembusu virus SD/02 strain; (C) Negative control.

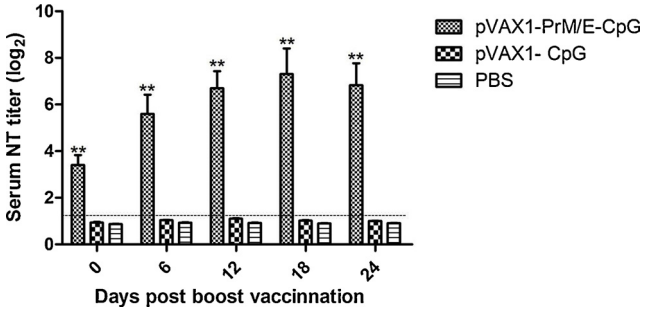

Fig. 3. Mean neutralizing antibody titers of ducks vaccinated with different immunological stimulants. Neutralizing antibody titers of ducks were tested using a virus neutralization test in BHK-21 cells. *denotes that the group had significantly different neutralizing antibody titers ($p < 0.05$) on the given days compared with the other groups; **denotes that the group had significantly different neutralizing antibody titers ($p < 0.01$) on the given days compared with the other groups.

3.5. Protection against virus challenge

To evaluate the efficiency of the DNA subunit vaccine to protect against TMUV infection, the survival and weight of ducks and clinical signs of the disease, e.g. paralysis, depression, acute anorexia, tilted head, and green and white diarrhea, were recorded for 15 days (Tables 1 and 2). In Group I ducks that were vaccinated with pVAX1-prM/E-CpG, no ducks died and only one duck exhibited mild symptoms (depression, green loose stools) at 3 days post infection (dpi), but quickly recovered the next day. The mortality rate of Group II was 10% (2/20), with mild neurological signs being observed in eight ducks until 7 dpi; two of these ducks died with severe neurological signs at 7 and 8 dpi. Post mortem examination of the dead ducks revealed enlarged spleens and livers and hemorrhage of the meninges and lungs. Similar observations were made for Group III. No mortality occurred in ducks from 9 dpi to the end of the study. The body weights of ducks in Group I were similar to that of those in Group IV, whereas the bodyweight of ducks in Groups II and III were significantly lower than those of Group IV (data not shown).

The Group I ducks that were challenged with TMUV exhibited short-term virus shedding, and viral nucleic acid could not be detected from 3 dpi to the end of the experiment. In contrast, ducks from Groups II and III that were challenged with TMUV continuously shed virus during the monitoring period.

4. Discussion

TMUV is an important pathogen that affects major duck breeding areas in China, but the current existing TMUV vaccines do not safely provide efficient protection against TMUV infection in ducks. In general, the inactivated vaccines that are frequently used on duck farms do not provide efficient protection against TMUV infection, and live vaccines can potentially be pathogenic for other flocks. In this study, we constructed a recombinant plasmid pVAX1-prM/E-CpG that can induce neutralizing antibodies against TMUV to protect ducks from infection and can be safely used as a candidate vaccine. This approach may have several potential advantages over other TMUV vaccine development strategies.

The use of the prM-E gene cassette as a DNA vaccine has been reported for different flaviviruses, such as Saint Louis encephalitis virus (Phillpotts et al., 1996), dengue serotype 1 and 2 viruses (Fonseca et al., 1994; Konishi et al., 2000a), Murray Valley encephalitis virus (Colombage et al., 1998), Japanese encephalitis virus (Konishi and Mason, 1993), Russian spring-summer encephalitis and tick-borne

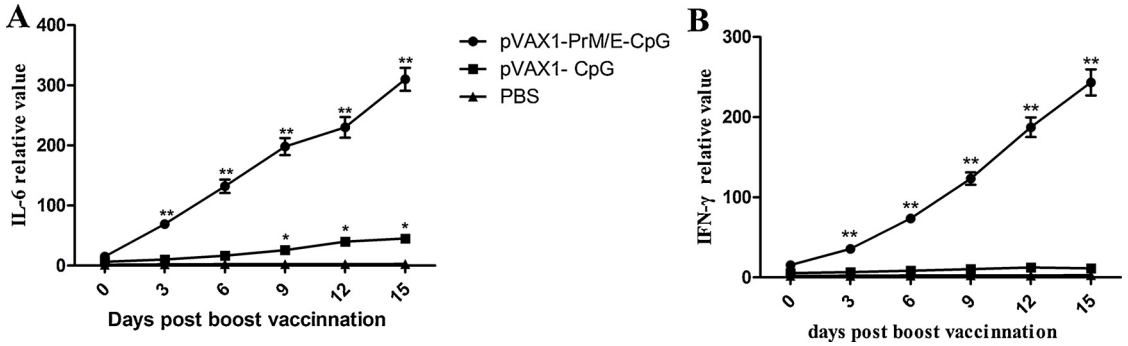

Fig. 4. Fold changes in expressions of IFN-γ and IL-6 genes in each group assessed using qRT-PCR. **denotes very significant difference ($p < 0.01$) in IFN-γ or IL-6 mRNA expressions compared with other groups. *denotes significant difference ($p < 0.05$) in IFN-γ or IL-6 mRNA expressions compared with other groups.

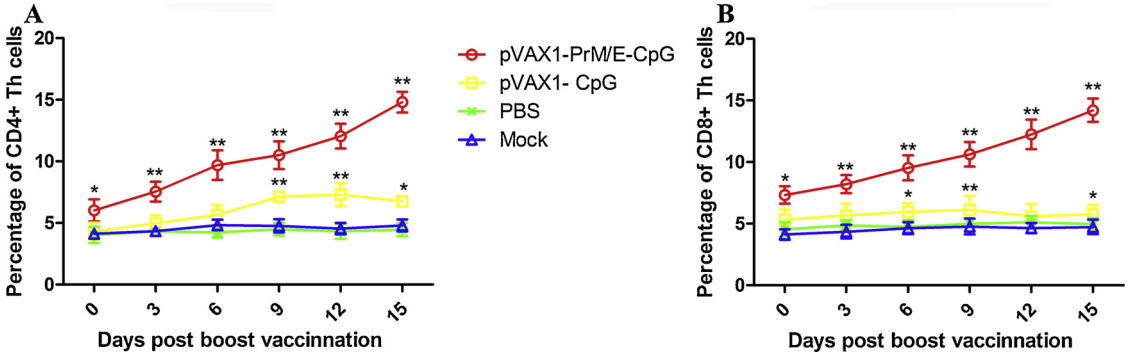

Fig. 5. $CD4^+$ and $CD8^+$ T lymphocyte counts in PBMCs. Duck PBMCs were isolated on day 0, 3, 6, 9, 12 and 15 dpi, and the percentages of $CD4^+$ and $CD8^+$ T lymphocytes were determined by flow cytometric analysis (n = 3). **denotes very significant difference ($p < 0.01$) compared with $CD4^+$ and $CD8^+$ T lymphocyte counts in other groups during the monitoring period. *denotes significant difference ($p < 0.05$) compared with $CD4^+$ and $CD8^+$ T lymphocyte counts in other groups during the monitoring period.

Table 1
Mortality of four repetition groups at week 2 post challenged.

Groups	Weeks post challenge		Mortality rate (%)
	1	2	
pVAX1-PrM/E-CpG	0/20	0/20	0
pVAX1-CpG	1/20[a]	2/20[a]	10
PBS	2/20[a]	3/20[a]	15
Negative control	0/20	0/20	0

[a] All brain samples collected from dead ducks were TMUV-positive using the semi-nested RT-PCR.

Central European encephalitis viruses (Schmaljohn et al., 1997). The recombinant DNA vaccine was based on prM and E genes which encode the major TMUV surface proteins that contain the principal neutralizing epitopes. It is proposed that virus neutralizing antibodies are important in humoral immunity against flavivirus infections. Maternal antibodies can effectively protect progeny animals so passively transferred monoclonal neutralizing antibodies would protect animals from lethal virus challenge. The prM gene was used in our DNA vaccine constructs because epitopes recognized by neutralizing antibodies have been identified in all three domains of the E protein, and studies with other flaviviruses have revealed that maintaining the proper conformation of E is associated with co-expression of the prM protein (Beltramello et al., 2010; Konishi et al., 2006; Martin et al., 2007). DNA has multiple and multifaceted effects on the immune system. The CpG-ODN containing three copies of the GACGTT motif was confirmed to enhance the humoral immune response and lymphocyte proliferation in specific-pathogen-free (SPF) chickens (Dalloul et al., 2004; Gomis et al., 2003). Therefore, we developed a recombinant construct containing the TMUV prM and E genes, with a CpG-ODN motif as an adjuvant.

We determined the immunogenicity of the TMUV DNA vaccine constructed and tested its protective efficacy in ducks following intramuscular vaccination. Both antibodies and cytotoxic T lymphocytes (CTLs) are deemed to be responsible for protection against viral infection (Bachmann et al., 1997). We demonstrated that ducks vaccinated with TMUV prM/E were protected against a lethal dose of Tembusu virus. The results of the antibody response suggest that vaccination with the DNA subunit vaccine may protect ducks against lethal TMUV infection. Vaccination of ducks induced a strong and long-lasting antibody response. The results of the TMUV virus challenge suggest that vaccination with the DNA subunit vaccine could provide effective protection against TMUV infection, although not a complete protection (one duck exhibited very mild symptoms). In field cases, TMUV infection principally occurs in ducks that are approximately 20 days old. Vaccination with TMUV DNA vaccine could induce circulating antiviral antibodies until the duck is slaughtered at 38 days old.

Cytokines are important in virus-induced immunopathology. Viral infection induces pro-inflammatory cytokine responses that are necessary to inhibit virus proliferation, including IL-6, IFN-γ, and other cytokines. IFN-γ and IL-6 transcription levels increased significantly in PBMCs isolated from ducks of all TMUV-challenged groups at 6, 8, and 10 dpi. After challenge with TMUV, transcription of IFN-γ in the DNA vaccine group (Group I) was still high compared with the negative control group (Group I). The up-regulation of IL-6 and IFN-γ in response to TMUV infection suggests that they are important in the protective immune response against this virus.

In conclusion, a DNA vaccine pVAX1-prM/E-CpG, which expressed the TMUV prM-E proteins both in vivo and in vitro, was successfully constructed. This DNA vaccine was shown to elicit antibody-mediated humoral immunity and cellular-associated immune responses, which are important in the course of TMUV infection and provide effective protection against lethal TMUV challenge. Previous studies have demonstrated that CpG-ODN enhances vaccine-induced immune responses in ducks (Lee et al., 2010). Therefore, the functional capacity of CpG-ODN was not tested or verified in our study. A supplementary study may be necessary to improve the efficacy of the DNA vaccine by optimizing certain parameters, such as the dose, vaccination days, and adjuvants used to improve the efficacy of the DNA vaccine. The results of our study indicate that the pVAX1-prM/E-CpG subunit vaccine may be an efficient candidate vaccine for the prevention and control of TMUV infection.

Table 2
The severity of clinical symptoms in ducks of TMUV inoculation groups at week 2 post infection.

Clinical changes	pVAX1-PrM/E-CpG	pVAX1- CpG	PBS
Depression	1/20 +	6/20 +, 4/20 + + +	5/20 +, 5/20 + + +
Titled head	1/20 +	4/20 + +, 2/20 + + +	3/20 + +, 2/20 + + +
Paralysis	0/20	5/20 + +, 3/20 + + +	5/20 + +, 3/20 + + +
Marasmus	0/20	3/20 +, 4/20 + +, 4/20 + + +	3/20 +, 5/20 + +, 2/20 + + +

Severity of clinical symptoms: + means mild symptoms; + + means moderate symptoms; + + + means severe symptoms.

Author contributions

Conceived and designed the experiments: HC, YT, YD. Performed the experiments: HC, YT, JQ, MY. Analyzed the data: HC, JQ. Contributed reagents/materials/analysis tools: YD, YT. Wrote the paper: HC, YT.

Acknowledgments

This study was funded by the National Natural Science Foundation of China (31272583, 31472199), the China Agriculture Research System (CARS-43-10B).

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