Heterologous prime-boost: an important candidate immunization strategy against tembusu virus

Yuting Pan
Sichuan Agricultural University - Chengdu Campus
Renyong Jia (jiary@sicau.edu.cn)
Avian Disease Research Center, Institute of Preventive Veterinary Medicine, Key Laboratory of Animal Disease and Human Health of Sichuan Province
https://orcid.org/0000-0002-3539-9184
Juping Li
Sichuan Agricultural University
Mingshu Wang
Sichuan Agricultural University
Shun Chen
Sichuan Agricultural University
Mafeng Liu
Sichuan Agricultural University
Dekang Zhu
Sichuan Agricultural University
Xinxin Zhao
Sichuan Agricultural University
Ying Wu
Sichuan Agricultural University
Qiao Yang
Sichuan Agricultural University
Zhongqiong Yin
Sichuan Agricultural University
Bo Jing
Sichuan Agricultural University
Juan Huang
Sichuan Agricultural University
Shaqiu Zhang
Sichuan Agricultural University
Lin Zhang
Sichuan Agricultural University
Yunya Liu
Sichuan Agricultural University
Yanlin Yu
Sichuan Agricultural University
Bin Tian
Sichuan Agricultural University
Leichang Pan
Sichuan Agricultural University
Mujeeb Ur Rehman
Sichuan Agricultural University
Anchun Cheng
Sichuan Agricultural University

Research

Keywords: Tembusu virus, E protein, vaccine, prime-boost strategy

Posted Date: January 24th, 2020

DOI: https://doi.org/10.21203/rs.2.21818/v1

Version of Record: A version of this preprint was published at Virology Journal on May 12th, 2020. See the published version at https://doi.org/10.1186/s12985-020-01334-w.

Abstract

Background

Tembusu virus (TMUV), a newly emerging pathogenic flavivirus, is acute and spreads rapidly which causes massive economic losses in the Chinese duck industry. Vaccination is the most effective method to prevent TMUV. So, it's urgent to look for an effective vaccine strategy against TMUV. Heterologous prime-boost regimen priming with DNA vaccine and boosting with recombinant adenovirus vaccine have been proven to be the successful strategy in protecting against virus in experimental animal models.

Methods

In this study, heterologous and homologous prime-boost strategies using DNA vaccine and recombinant adenovirus vaccine expressing prM-E or E protein of TMUV were evaluated to protect ducks against the infection of TMUV for the first time, including priming and boosting with DNA vaccine, priming and boosting with recombinant adenovirus vaccine, and priming with DNA vaccine and boosting with recombinant adenovirus vaccine. Humoral and cellular immune responses were detected and evaluated. We then challenged the ducks with TMUV at 12 days after boosting to assay for the clinical symptoms, mortality, viral loads and histopathological lesions of these different strategies.

Results

Comparing with homologous prime-boost strategies, higher levels of specific antibodies against E protein and the neutralizing antibodies against TMUV were detected in heterologous prime-boost regimen. And also, it could induce higher levels of IFN-γ than homologous prime-boost strategies in the later stage. Interestingly, heterologous prime-boost strategy induced higher level of IL-4 in the early stage, but gradually decreased and was even lower than homologous prime-boost strategy in the later stage. Moreover, the heterologous prime-boost strategy could efficiently protect ducks with low viral tiers, no clinical symptoms and histopathological lesions in this experiment after challenging with TMUV while slight clinical symptoms and histopathological lesions were observed in homologous prime-boost strategies.

Conclusions

Our results indicated that the heterologous prime-boost strategy induced higher levels of humoral and cellular immune responses and better protection against the TMUV infection in ducks than the homologous prime-boost strategies, suggesting that heterologous prime-boost strategy is an important candidate for the design of a novel vaccine strategy against TMUV.

Background

The genus flavivirus is single-stranded RNA virus which composed of more than 70 viruses, such as tembusu virus (TMUV) [1], tick-borne encephalitis virus (TBEV) [2], dengue virus (DENV) [3], West Nile virus (WNV) [4], Japanese encephalitis virus (JEV) [5] and zika virus (ZIKV) [6]. Flavivirus can encode three structural proteins: capsid (C), pre-membrane/membrane (prM/M) and envelope (E), and seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. These proteins have extremely important roles in viral invasion, replication and regulation of the host factors. The flavivirus can spread between animals and humans, causing zoonotic diseases [7]. Because of the sharp increasing in global morbidity in the past few years, it has become a public health issue which people pay much attention.

TMUV is a newly emerging virus, which is characterized by growth slowly, decreased appetite, neurological dysfunction and a serious drop in egg production [8]. TMUV mainly infects ducks [9], chickens [10], goose [11], pigeons [12] and even sparrows [13]. Moreover, human may also be threatened by infecting TMUV [14]. Vaccination is the most effective method to prevent TMUV infection. Therefore, it is urgently required for an effective vaccine immunized strategy against TMUV. The E protein, which is the major antigenic determinant of the three structural proteins, contains many neutralizing epitopes and plays a critical role in the host cell entry-attachment to cellular receptors and membrane fusion. And also, E protein is the key region of viral virulence with great immunogenicity which can induce immune protection effectively [15], suggesting that the E protein can be used as a vaccine candidate against TMUV. The prM protein, which is regarded as the chaperone protein of E, can assist E protein in proper folding and assembly, and protects the structural stability of the E protein. Duck IL-2, a gene adjuvant of vaccine, can strengthen the antigen-specific immune response of the vaccine and induce a highly effective immunogenicity, which can provide the body with a more comprehensive and efficient immune protection [16].

Homologous prime-boost regimen is used in traditional vaccine, but better preventive effects have been reported of the infectious diseases by heterologous prime-boost strategy, which consists of DNA vaccine priming followed by recombinant adenovirus boosting [17]. The heterologous prime-boost strategy can induce strong humoral and cellular immune responses [18, 19].

In the present studies, vaccine strains using Salmonella-presented TMUV prM-E gene [20] and recombinant adenovirus packaging TMUV E gene with IL-2 as the vaccine adjuvant have been successfully constructed. We wonder if the heterologous prime-boost regimen (priming with DNA vaccine and boosting with recombinant adenovirus) is more effective than the homologous prime-boost strategies (priming and boosting with DNA vaccine; priming and boosting with recombinant adenovirus vaccine). The results showed that the heterologous prime-boost strategy could induce higher levels of specific antibodies and better protection against the TMUV infection than the homologous prime-boost regimens. Therefore, heterologous prime-boost strategy should be carefully considered to induce the desired immune response.

Methods

Virus and vaccines

The TMUV (CQW strain) (GenBank: KM233707.1) used in this study was provided by Research Center of Avian Disease of Sichuan Agricultural University. The virus was propagated in the allantoic cavities of 10-day-old specific pathogen-free (SPF) embryonated duck eggs. Vaccine strains using Salmonella-presented TMUV prM-E gene (SE) and recombinant adenovirus packaging TMUV E gene with IL-2 as the vaccine adjuvant (ads) were saved in our lab. The positive control was purchased from Qilu Animal Health company.

Animal Experiment

Ducklings (one-day-old) (n = 170) were purchased from standard farm in Yaan and randomly divided into 5 groups as shown in Table 1. All ducklings were obtained from flocks with no prior exposure to TMUV, and were immunized twice at 7-day-old and 19-day-old, respectively. At 3, 10, 17, 24, 31, 38, 48 and 60 days post prime injection (dpi), three ducks' serum and spleen from each group were collected randomly and stored in -80℃ (Fig. 1). 12 days after boosting, ducks (n = 10) of each group were randomly selected and challenged with 1 ml $10^{5.1}$-fold 50% of embryo lethal death (ELD_{50}) TMUV per duck by intramuscular injection (i.m.). The clinical symptoms and death were recorded until 7 days after challenging. Then, the heart, liver, spleen, kidney and brain of the ducks were collected and stored at 7 days after challenging (Fig. 1).

Neutralization Assay

Sera samples from the immunized ducks were collected at 3, 10, 17, 24, 31, 38, 48 and 60 dpi, and inactivated with heat at 56℃ for 1 h (n = 3 for each time point) and serial 2-fold diluted to 2^{-8} with phosphate buffer saline, and then mixed with an equal volume of 100-fold 50% tissue culture infective dose ($TCID_{50}$) of TMUV and incubated in duck embryo fibroblast (DEF) cells with 5% CO_2 at 37℃ for 1 h, then removed the mixture and added Dulbecco's modified eagle media (DMEM) with 2% serum. The cells were cultured at 37℃ with 5% CO_2 and observed to record the cytopathic effect (CPE) for at least five days.

Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was used to detect the IgG antibody titers in the collecting sera. TMUV E protein antigen (100 μl per well) as the capture molecular was coated in the 96-well plates and incubated overnight at 4℃. After washing the 96-well plates three times with phosphate buffer saline with Tween (PBST), the plates were blocked with 1% BSA for 1 h at 37℃. Horseradish peroxidase (HRP)-conjugated goat anti-duck IgG antibody (Solarbio, China) was used as the secondary antibody at a 1:2000 dilution for 1 h at 37℃. After washing the plates three times with PBST, tetramethylbenzidine (TMB) (100 μl per well) was added in the plates lucifugally for 10 min, and the reaction was stopped by 100 μl 2 M H_2SO_4. Then, the optical density was measured in each sample at 450 nm.

Quantification Of Cytokines

Total RNA of spleen collected from immunized ducks was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized from 1000 ng RNA using PrimeScript II 1st Strand cDNA Synthesis Kit with oligo dT primers (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. Then an equal volume of cDNA was then subjected to quantitative real-time polymerase chain reaction (RT-PCR) using SYBR Green Real-Time RT-PCR Master Mix Plus (Promega) and primers. The primers of cytokine genes and β-action used in this study were listed in Table 2. The procedure of quantitative RT-PCR was performed as previous work [21]. The results were analyzed by the Bio-Rad CFX Manager Software and Graphpad 7.0. by $2^{-\Delta\Delta Ct}$ method and expressed as the mean ± standard deviation.

Detection of viral loads in challenging ducks by quantitative RT-PCR

Viral loads in tissues (heart, liver, spleen, kidney and brain) were detected by quantitative RT-PCR according to the method established previously [22]. Primers of TMUV C protein were listed in Table 2. The results were analyzed by the Bio-Rad CFX Manager Software and Graphpad 7.0. by $2^{-\Delta\Delta Ct}$ method and expressed as the mean ± standard deviation.

Pathological Identification And Histological Examination

The tissues (heart, liver, spleen, kidney and brain) were fixed with 4% paraformaldehyde solution for at least 48 h, then embedded in paraffin, and sliced into 4 μm sections (Leica RM2128, Wetzlar, Germany). The tissue sections were deparaffinized with xylene and rehydrated with gradient ethanol. Then the sections were stained by haematoxylin and eosin (Solarbio, Beijing, China) and counterstained with hematoxylin (Solarbio, Beijing, China), and then dehydrated through series gradient ethanol solutions. We performed the observation with microscope (Olympus BX43, Tokyo, Japan).

Statistical Analysis

Data and imaging were all performed by Graphpad 7.0. The relative mRNA expression was expressed as the mean ± standard deviation and analyzed by $2^{-\Delta\Delta Ct}$ method. The statistical significance was assessed by t-test. *p<0.05 indicates significance.

Results

Specific antibody responses

In order to determine the protective immune responses induced by the heterologous or homologous prime-boost strategies, the neutralizing antibodies were measured by neutralizing assay at 3, 10, 17, 24, 31, 38, 48 and 60 dpi. The neutralizing antibody levels of ads/ads and SE/SE group gradually increased and reached to the peak at 38 dpi and 48 dpi with 5.7 log_2-folds and 6.2 log_2-folds (Fig. 2A). As for SE/ads group, the level of neutralizing antibody reached to the

peak at 38 dpi with 6.9 log_2-folds, and maintained a high level after the peak. The peak of SE/ads group was 2.4 and 1.4 times higher than those of ads/ads and SE/SE groups, respectively. And the overall level of neutralizing antibodies in SE/ads was higher than that of the ads/ads and SE/SE groups. As for the WF/WF group, the levels of neutralizing antibodies reached to the peak at 31 dpi with 6.4 log_2-folds, but decreased quickly after the peak. There were no neutralizing antibody responses in the serum of ducks from PBS/PBS group (Fig. 2A).

A high level of specific antibodies against E protein in SE/ads was observed, which showed a 5.6-fold higher level at 38 dpi compared with the PBS/PBS group (Fig. 2B). And the antibodies in SE/ads group remained at high levels from 38 to 60 dpi and were higher than that in ads/ads and SE/SE from 24 to 60 dpi. As for the WF/WF, the titers of antibodies reached to the peak at 31 dpi with 4.93-fold higher than that in PBS/PBS group, but decreased quickly after the peak, and even lower than that in SE/ads after 31dpi (Fig. 2B).

The results herein suggested that the heterologous prime-boost strategy could efficiently induce the production of higher titers of antibodies in ducks than the homologous prime-boost strategies.

Cytokines induced by the difference immune strategies after immunization

To further characterize the induced immune response, the quantitative RT-PCR was used to measure the cytokines (IFN-γ and IL-4) in the spleen. The expression level of IFN-γ in ads/ads and SE/SE reached to the peak with 10.7-fold and 4.7-fold higher at 38 dpi and 31dpi. As for the SE/ads, the expression level of IFN-γ increased quickly and exceeded the other three groups after 31dpi. Then it reached to the peak at 48 dpi with 16.4-fold higher and maintained a high level of IFN-γ from 38 to 60 dpi. The expression level of IFN-γ in WF/WF, which reached to the peak at 31 dpi with 11.1-fold higher, was generally higher than that in ads/ads and SE/SE, and lower than that in SE/ads after 31 dpi (Fig. 3A).

As for the IL-4, the expression levels of IL-4 in ads/ads and SE/SE both reached to the peak with 11.5-fold and 16.8-fold higher at 38 dpi, and the expression levels were lower than that in SE/ads from 17 to 31dpi. Interestingly, the expression levels of IL-4 in SE/ads reached to the peak at 31 dpi with 16.4-fold higher, but slowly decreased after the peak, and the levels was even lower than that in SE/SE after 31 dpi. As for WF/WF, the expression level of IL-4 was generally higher than that in SE/SE and SE/ads groups, but lower than that in ads/ads after 31 dpi (Fig. 3B).

Collectively, these results demonstrated that the heterologous prime-boost strategy resulted in strong stimulation of IFN-γ (in the later stage) and IL-4 (in the early stage) after immunization than the homologous prime-boost strategies, which indicated that the heterologous prime-boost strategy could induce better cellular immune response against TMUV.

Protection of ducks against TMUV after challenging

To verify the clinical protection of ducks against TMUV infection, the immunized ducks were challenged with 1 ml $10^{5.1}$-fold ELD_{50} TMUV at 12 days after the boost vaccination (Table 1). All the ducks in ads/ads, SE/ads and WF/WF groups were survived. However, 10% and 30% ducks in SE/SE and PBS/PBS groups died after challenging (Fig. 4A). Thus, the survival ratio in SE/SE and PBS/PBS were 90% and 70%, while the survival ratio in ads/ads, SE/ads and WF/WF were 100%. In order to compare the protection against TMUV of these groups, the clinical signs were also recorded to evaluate the efficiency of these strategies. The ducks in PBS/PBS showed typical clinical signs after challenging, such as depression, decreasing appetite and neurological symptoms. However, slight clinical signs were also observed in ads/ads and SE/SE groups while no clinical signs were observed in SE/ads and WF/WF groups. These results indicated that the heterologous prime-boost strategy was with a better protection of the ducks against the challenging of TMUV than that of the homologous prime-boost strategies.

Viral loads in the tissues of ducks after challenging

The titers of TMUV were measured by quantitative RT-PCR based on the C gene of TMUV at 7 days after challenging in heart, liver, spleen, kidney and brain. The TMUV could be detected in all tissues from the five groups after challenging. The viral titers of the spleen were higher than that of the other tissues (heart, liver, kidney and brain) (Fig. 4B). The titers of TMUV in SE/ads was generally lower than that of ads/ads and SE/SE groups in heart, liver, spleen, kidney and brain. In heart, the titers of TMUV in SE/ads was significantly lower than that in ads/ads and SE/SE groups (P□0.01). In liver, spleen and brain, the viral titers in SE/ads were significantly lower than that in SE/SE group (P□0.01), but no significant difference with that in ads/ads groups. In kidney, the titers of TMUV in ads/ads, SE/SE and SE/ads had no significant difference with each other (Fig. 4B). To sum up, the heterologous and homologous prime-boost regimens could both inhibit the viral replication after challenging in ducks, but the heterologous prime-boost regimen could better prevent the viral replication than the homologous prime-boost strategies.

Histopathological Observation

The ducks were euthanized at 7 days after challenging, and their tissues (heart, liver, spleen, kidney and brain) were collected for histopathological observation. Histological observation with different groups showed different histopathological changes. In heart, slight myocardial fiber rupture and lymphocytic infiltration were found in the ads/ads and SE/SE groups (Fig. 5A and 5B), while no significant pathological damages were observed in SE/ads and WF/WF groups (Fig. 5C and 5D). But severe lesions in the PBS/PBS group were found with myocardial fiber rupture, edema and lymphocytic infiltration (Fig. 5E).

In liver, it showed slight hepatocyte vacuolation in ads/ads group (Fig. 5F). Hepatocyte vacuolation and lymphocyte cell infiltration were observed in SE/SE group (Fig. 5G), while the SE/ads and WF/WF groups had no obvious lesions (Fig. 5H and 5I). As for the PBS/PBS group, the liver revealed severe hepatocyte vacuolation, hepatocyte necrosis and massive lymphocyte infiltration (Fig. 5J).

In spleen, obvious decreasing lymphocytic, increasing reticulocyte and unclear boundary between red and white pulp were founded in ads/ads, SE/SE and PBS/PBS groups, while the other groups showed no significant lesions (Fig. 5K-5O).

In kidney, no obvious pathological changes were observed in the ads/ads, SE/ads and WF/WF groups (Fig. 5P, 5R and 5S). The basement membranes of renal epithelial cells in the SE/SE and PBS/PBS groups were detached obviously (Fig. 5Q and 5T). Moreover, the kidney also experienced bleeding and necrotic in the epithelial cells of the PBS/PBS group (Fig. 5T).

In brain, there were no obvious pathological changes in ads/ads, SE/ads and WF/WF groups (Fig. 5U, 5W and 5X) while observed. Vascular sleeve phenomenon occurred in the brain tissues of the SE/SE group while the PBS/PBS showed both vascular sleeve and satellite phenomenon (Fig. 5V and 5Y).

In summary, there were slight lesions in ads/ads and SE/SE groups, no microscopic lesions in SE/ads and WF/WF groups, but the most severe lesions in PBS/PBS group.

Discussion

Since 2010, TMUV has caused huge economic losses in China. Studies have shown that TMUV can infect ducks, chickens, geese, and mice. Thus, development of vaccines against TMUV is urgent. Many prototype vaccines against TMUV have been developed, such as liposome containing recombinant E protein vaccine [23], live attenuated vaccine against TMUV [24] and recombinant duck enteritis virus expressing E protein of TMUV [25]. Traditionally, the prime-boost strategy is performed using the same vaccine. However, new studies have suggested that the prime-boost strategy can be performed with different vaccines expressing the same antigen [26–29]. This type of heterologous prime-boost strategy has been used extensively in the studies of vaccines against lots of pathogens including human immunodeficiency virus (HIV), hepatitis C virus (HCV), pseudorabies virus and herpesvirus [30–32]. This may suggest that the heterologous prime-boost regimen can confer synergistically stronger responses to antigens and greater protection than immunized with the same vaccine alone [33, 34].

Based on our lab, vaccine strains using Salmonella-presented TMUV prM-E gene and recombinant adenovirus packaging TMUV E gene have been constructed. This paper describes the successful use for the first time in flavivirus of a heterologous prime-boost combination of Salmonella presented TMUV prM-E gene and recombinant adenovirus packaging TMUV E gene to immunize ducks against TMUV infection.

Currently, the available vaccines of flavivirus against JEV, DENV and TBEV infections can induce high levels of neutralizing antibody which is deemed as a significant for protection after virus infection. The titers of neutralizing antibody are considered as a surrogate marker in vaccine evaluation [35]. In this study, the levels of antibodies were detected after immunizing with heterologous or homologous prime-boost strategies in ducks. The results showed that the heterologous prime-boost strategy could induce stronger antibody immunization compared with the homologous prime-boost regimens, especially after 31 dpi. This phenomenon also existed in neutralizing antibodies. The high levels of antibodies could greatly neutralize the free TMUV in the host and prevented the virus from copying and spreading in the cells.

One interesting thing was that the cytokines of the heterologous prime-boost strategy was higher than that of the homologous prime-boost strategies in the early stage (17-31dpi) in IL-4 level (Th2 biased cytokine), and the later stage (after 31dpi) in IFN-γ level (Th1 biased cytokine), this may be the reason of the presence of Th1-Th2 balance. The expression levels of IFN-γ in SE/ads group was inhibited by the high levels of IL-4 in the early stage while the IL-4 level in SE/ads group was inhibited by the high levels of IFN-γ in the later stage. Thus, the IFN-γ levels in SE/ads group increased slowly in the early stage while the IL-4 levels decreased slowly in the later period. But the levels of IL-4 in SE/ads were always higher than that of ads/ads in the eight time points. Overall, in the early stage, there was no significant difference in the expression level of IFN-γ between the heterologous or homologous prime-boost strategies. But in the later stage, the heterologous prime-boost strategy could cause higher levels of IFN-γ than the homologous prime-boost regimens, which could trigger a stronger cellular immune response and better protect the ducks against TMUV.

Based on the protection against TMUV challenging, the ducks in ads/ads group were all survived after challenging. However, there were still slight clinical signs and lesions in heart, liver and spleen while observed under microscope. As for the SE/SE, 90% survival rate in ducks was recorded after challenging with infecting TMUV and slight lesions observed in tissues suggesting that the SE/SE could only confer partial protection against TMUV. The results indicated that heterologous prime-boost strategy was a better regimen than the homologous prime-boost regimens, as SE/ads could greatly inhibit TMUV replication in ducks and provided the complete protection against the TMUV infection with no death and no lesions in tissues, whereas SE/SE and ads/ads could only confer partial protection when vaccinated ducks after challenging.

The presented heterologous prime-boost regimen can be regarded as an alternative anti-TMUV vaccine strategy, as high titers of antibodies in the blood and all vaccinated animals survived with no clinical signs and no lesions in tissues after challenging. Discrimination between vaccinated and field-infected animals is possible due to the use of prM and E proteins only. Finally, in our study, the heterologous prime-boost regime generated no lesions in ducks, but further studies are still needed to prove the safety of this regimen in ducks.

In conclusion, this study described the first attempt to a novel immune strategy of heterologous prime-boost regimen based on the Salmonella-presented TMUV prM-E gene and recombinant adenovirus packaging TMUV E gene. The results showed that the heterologous prime-boost strategy could form great immunogenicity, and protected the ducks from the threating of TMUV infection. So, the heterologous prime-boost regimen has a great potential as a preventive strategy against TMUV infection.

Conclusions

The heterologous prime-boost strategy could induce higher levels of antibodies and better protection against the TMUV infection in ducks than the homologous prime-boost strategies, suggesting that the heterologous prime-boost strategy is an important candidate for the design of a novel vaccine strategy against TMUV.

Abbreviations

TMUV:tembusu virus; TBEV:tick-borne encephalitis virus; DENV:dengue virus; WNV:West Nile virus; JEV:Japanese encephalitis virus; ZIKV:zika virus; C:capsid; prM/M:pre-membrane/membrane; E:envelope; NS:non-structural; IL-2:interleukin-2; SPF:specific pathogen-free; ads:recombinant adenovirus packaging TMUV E gene with IL-2 as the vaccine adjuvant; SE:Salmonella-presented TMUV prM-E gene; WF:positive vaccine named WF100 purchased from Qilu Animal Health company; PBS:negative control injected ducks with phosphate buffer saline; ads/ads:priming and boosting with ads; SE/SE:priming and boosting with SE; SE/ads:priming with SE and boosting with ads; WF/WF:priming and boosting with WF; PBS/PBS:priming and boosting with PBS; dpi:days post prime injection; ELD50:50% of embryo lethal death; i.m.:intramuscular injection; TCID50:50% tissue culture infective dose; DEF:duck embryo fibroblast; DMEM:Dulbecco's modified eagle media; CPE:cytopathic effect; ELISA:enzyme-linked immunosorbent assay; IL-4:interleukin-4; IFN-γ:interferon-γ; RT-PCR:real-time polymerase chain reaction; PBST:phosphate buffer saline with Tween; HIV:human immunodeficiency virus; HCV:hepatitis C virus

Declarations

Ethics approval and consent to participate

This animal study was approved by the Animal Ethics Committee of Sichuan Agricultural University (approval No. XF2016-17), China. Experiments were conducted in accordance with guidelines and regulations of National Institutes of Health.

Consent for publication

Not applicable.

Availability of data and materials

The data sets analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China (31872475), Sichuan Veterinary Medicine and Drug Innovation Group of China Agricultural Research System (CARS-SVDIP), China Agricultural Research System (CARS-42-17), and Sichuan Province Research Programs (2017JY0014).

Authors' contributions

YP, RJ and JL conceived and designed the experiments; YP and JL performed the experiments; YP analyzed the data; YP, JL revised the manuscript; MW, SC, ML, DZ, XZ, YW, QY, ZY, BJ, JH, SZ, LZ, YL, YY, BT, LP, MN and AC helped in the experiments. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

Author details

[1]Research Center of Avian Disease, College of Veterinary Medicine of Sichuan Agricultural University, Wenjiang 611130, P.R. China; [2]Key Laboratory of Animal Disease and Human Health of Sichuan Province, Wenjiang 611130, P.R. China; [3] Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Wenjiang 611130, P.R. China.

References

1. Thontiravong A, Ninvilai P, Tunterak W, et al. Tembusu-Related Flavivirus in Ducks, Thailand. Emerg Infect Dis. 2015; 21(12):2164-7.
2. Goto A, Yoshii K, Obara M, et al. Role of the N-linked glycans of the prM and E envelope proteins in tick-borne encephalitis virus particle secretion. Vaccine. 2005; 23(23):3043-52.
3. Mustafa MS, Rasotgi V, Jain S, et al. Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. Med J Armed Forces India. 2015; 71(1):67-70.
4. Jimenez de Oya N, Escribano-Romero E, Camacho MC, et al. A recombinant subviral particle-based vaccine protects magpie (Pica pica) against West Nile virus infection. Front Microbiol. 2019; 10:1133-43.
5. Kim JM, Yun SI, Song BH, et al. A single N-linked glycosylation site in the Japanese encephalitis virus prM protein is critical for cell type-specific prM protein biogenesis, virus particle release, and pathogenicity in mice. J Virol. 2008; 82(16):7846-62.

6. Agrelli A, de Moura RR, Crovella S, et al. ZIKA virus entry mechanisms in human cells. Infect Genet Evol. 2019; 69:22-29.
7. Zhang X, Jia R, Shen H, et al. Structures and functions of the envelope glycoprotein in flavivirus infections. Viruses. 2017; 9(11):338-52.
8. Huang J, Shen H, Jia R, et al. Oral vaccination with a DNA vaccine encoding capsid protein of duck Tembusu virus induces protection immunity. Viruses. 2018; 10(4):180-92.
9. Li N, Lv C, Yue R, et al. Effect of age on the pathogenesis of duck tembusu virus in Cherry Valley ducks. Front Microbiol. 2015; 6:581.
10. Liu M, Chen S, Chen Y, et al. Adapted Tembusu-like virus in chickens and geese in China. J Clin Microbiol. 2012; 50(8):2807-09.
11. Huang X, Han K, Zhao D, et al. Identification and molecular characterization of a novel flavivirus isolated from geese in China. Res Vet Sci. 2013; 94(3):774-80.
12. Liu P, Lu H, Li S, et al. Genomic and antigenic characterization of the newly emerging Chinese duck egg-drop syndrome flavivirus: genomic comparison with Tembusu and Sitiawan viruses. J Gen Virol. 2012; 93(Pt_10):2158-70.
13. Tang Y, Diao Y, Yu C, et al. Characterization of a Tembusu virus isolated from naturally infected house sparrows (Passer domesticus) in Northern China. Transbound Emerg Dis. 2013; 60(2):152-58.
14. Tang Y, Gao X, Diao Y, et al. Tembusu virus in human, China. Transbound Emerg Dis. 2013; 60(3):193-96.
15. Wu KP, Wu CW, Tsao YP, et al. Structural basis of a flavivirus recognized by its neutralizing antibody: Solution structure of the domain III of the Japanese encephalitis virus envelope protein. J Biol Chem. 2003; 278(46):46007-13.
16. Zhang HY, Sun SH, Guo YJ, et al. Optimization strategy for plasmid DNAs containing multiple-epitopes of foot-and-mouth disease virus by cis-expression with IL-2. Vaccine. 2008; 26(6):769-77.
17. Li J, Yang T, Xu Q, et al. DNA vaccine prime and recombinant FPV vaccine boost: An important candidate immunization strategy to control bluetongue virus type 1. Appl Microbiol Biotechnol. 2015; 99(20):8643-52.
18. Kardani K, Bolhassani A, Shahbazi S. Prime-boost vaccine strategy against viral infections: Mechanisms and benefits. Vaccine. 2016; 34(4):413-23.
19. Lu S. Heterologous prime-boost vaccination. Curr Opin Immunol. 2009; 21(3):346-51.
20. Huang J, Jia R, Shen H, et al. Oral delivery of a DNA vaccine expressing the PrM and E Genes: A promising vaccine strategy against flavivirus in ducks. Sci Rep. 2018; 8(1):12360.
21. Qi Y, Chen S, Zhao Q, et al. Molecular cloning, tissue distribution, and immune function of goose TLR7. Immunol Lett. 2015; 163(2):135-42.
22. Zhang X, Jia R, Pan Y, et al. Therapeutic effects of duck Tembusu virus capsid protein fused with staphylococcal nuclease protein to target Tembusu infection in vitro. Vet Microbiol. 2019; 235:295-300.
23. Ma T, Liu Y, Cheng J, et al. Liposomes containing recombinant E protein vaccine against duck Tembusu virus in ducks. Vaccine. 2016; 34(19):2157-63.
24. Li G, Gao X, Xiao Y, et al. Development of a live attenuated vaccine candidate against duck Tembusu viral disease. Virology. 2014; 450-451:233-42.
25. Chen P, Liu J, Jiang Y, et al. The vaccine efficacy of recombinant duck enteritis virus expressing secreted E with or without PrM proteins of duck tembusu virus. Vaccine. 32(41):5271-77.
26. Gu Y, Zhan B, Yang Y, et al. Protective effect of a prime-boost strategy with the Ts87 vaccine against Trichinella spiralis infection in mice. Biomed Res Int. 2014; 2014:326860-9.
27. Rama Rao A, Francois V, John D. A, et al. Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Vaccine. 2002; 20(15):69-74.
28. Letvin NL, Huang Y, Chakrabarti BK, et al. Heterologous envelope immunogens contribute to AIDS vaccine protection in Rhesus monkeys. J Virol. 2004; 78(14):7490-97.
29. McConkey SJ, Reece WH, Moorthy VS, et al. Enhanced T-cell immunogenicity of plasmid DNA vaccines boosted by recombinant modified vaccinia virus Ankara in humans. Nat Med. 2003; 9(6):729-35.
30. Rollier C, Verschoor E, Paranhos B, et al. Modulation of vaccine-induced immune responses to Hepatitis C virus in rhesus macaques by altering priming before adenovirus boosting. J Infect Dis. 2005; 192(5):920-29.
31. Kim SJ, Kim HK, Han YW, et al. Multiple alternating immunizations with DNA vaccine and replication-incompetent adenovirus expressing gB of pseudorabies virus protect animals against lethal virus challenge. J Microbiol Biotechn. 2008; 18(7):1326-34.
32. Dory D, Fischer T, Béven V, et al. Prime-boost immunization using DNA vaccine and recombinant Orf virus protects pigs against Pseudorabies virus (Herpes suid 1). Vaccine. 2006; 24(37-39):6256-63.
33. Hong W, Xiao S, Rui Z, et al. Protection induced by intramuscular immunization with DNA vaccines of pseudorabies in mice, rabbits and piglets. Vaccine. 2002; 20(7-8):1205-14.
34. Passarinha LA, Diogo MM, Queiroz JA, et al. Production of ColE1 type plasmid by Escherichia coli DH5α cultured under non-selective conditions. J Microbiol Biotechn. 2006; 16(1):20-24.
35. Ishikawa T, Yamanaka A, Konishi E. A review of successful flavivirus vaccines and the problems with those flaviviruses for which vaccines are not yet available. Vaccine. 2014; 32(12):1326-37.

Tables

Table 1. Experimental designs of the animal studies. Experimental designs of the animal studies. The abbreviations in the table are as follows: ads: recombinant adenovirus packaging TMUV E gene with IL-2 as the vaccine adjuvant; SE: Salmonella-presented TMUV prM-E gene; WF: positive vaccine named WF100 purchased from Qilu Animal Health company; PBS: negative control injected ducks with phosphate buffer saline; ads/ads: priming with ads and

boosting with ads; SE/SE: priming with SE and boosting with SE; SE/ads: priming with SE and boosting with ads; WF/WF: priming with WF and boosting with WF; PBS/PBS: priming with PBS and boosting with PBS

Experiment purpose	Administration								
	groups	Number of animals/ducks	Prime (7 days)			Boost (19 days)			
			vaccine	Dosage	Route	vaccine	Dosage	Route	Challenge dosage
Evaluation of immunogenicity in ducks	ads/ads	24	ads	10^7PFU/0.5ml	Intramuscular	ads	10^7PFU/0.5ml	Intramuscular	-
	SE/SE	24	SE	10^{11}CFU/0.5ml	Oral injection	SE	10^{11}CFU/0.5ml	Oral injection	-
	SE/ads	24	SE	10^{11}CFU/0.5ml	Oral injection	ads	10^7PFU/0.5ml	Intramuscular	-
	WF/WF	24	WF	0.5ml	Intramuscular	WF	0.5ml	Intramuscular	-
	PBS/PBS	24	PBS	0.5ml	Intramuscular	PBS	0.5ml	Intramuscular	-
Protection effect	ads/ads	10	ads	10^7PFU/0.5ml	Intramuscular	ads	10^7PFU/0.5ml	Intramuscular	$10^{5.1}ELD_{50}$
	SE/SE	10	SE	10^{11}CFU/0.5ml	Oral injection	SE	10^{11}CFU/0.5ml	Oral injection	$10^{5.1}ELD_{50}$
	SE/ads	10	SE	10^{11}CFU/0.5ml	Oral injection	ads	10^7PFU/0.5ml	Intramuscular	$10^{5.1}ELD_{50}$
	WF/WF	10	WF	0.5ml	Intramuscular	WF	0.5ml	Intramuscular	$10^{5.1}ELD_{50}$
	PBS/PBS	10	PBS	0.5ml	Intramuscular	PBS	0.5ml	Intramuscular	$10^{5.1}ELD_{50}$

Table 2. List of primers and sequences in this study

Primer names	Polarity	Sequence (5’ - 3’)	Reference
IFN-γ(f)	Forward	CATACTGAGCCAGATTGTTACCC	New
IFN-γ(r)	Reverse	TCACAGCCTTGCGTTGGA	
IL-4(f)	Forward	TCTATCAGAGAAAGACAACAC	New
IL-4(r)	Reverse	GGTGACTATTTCTTTCAAGT	
β-action(f)	Forward	CCGTGACATCAAGGAGAA	[21]
β-action(r)	Reverse	GAAGGATGGCTGGAAGAG	
TMUV-C(f)	Forward	AGGTTTGTGCTGGCTCTAC	[22]
TMUV-C(r)	Reverse	TGTTTGGTCGCCTCATT	

Figures

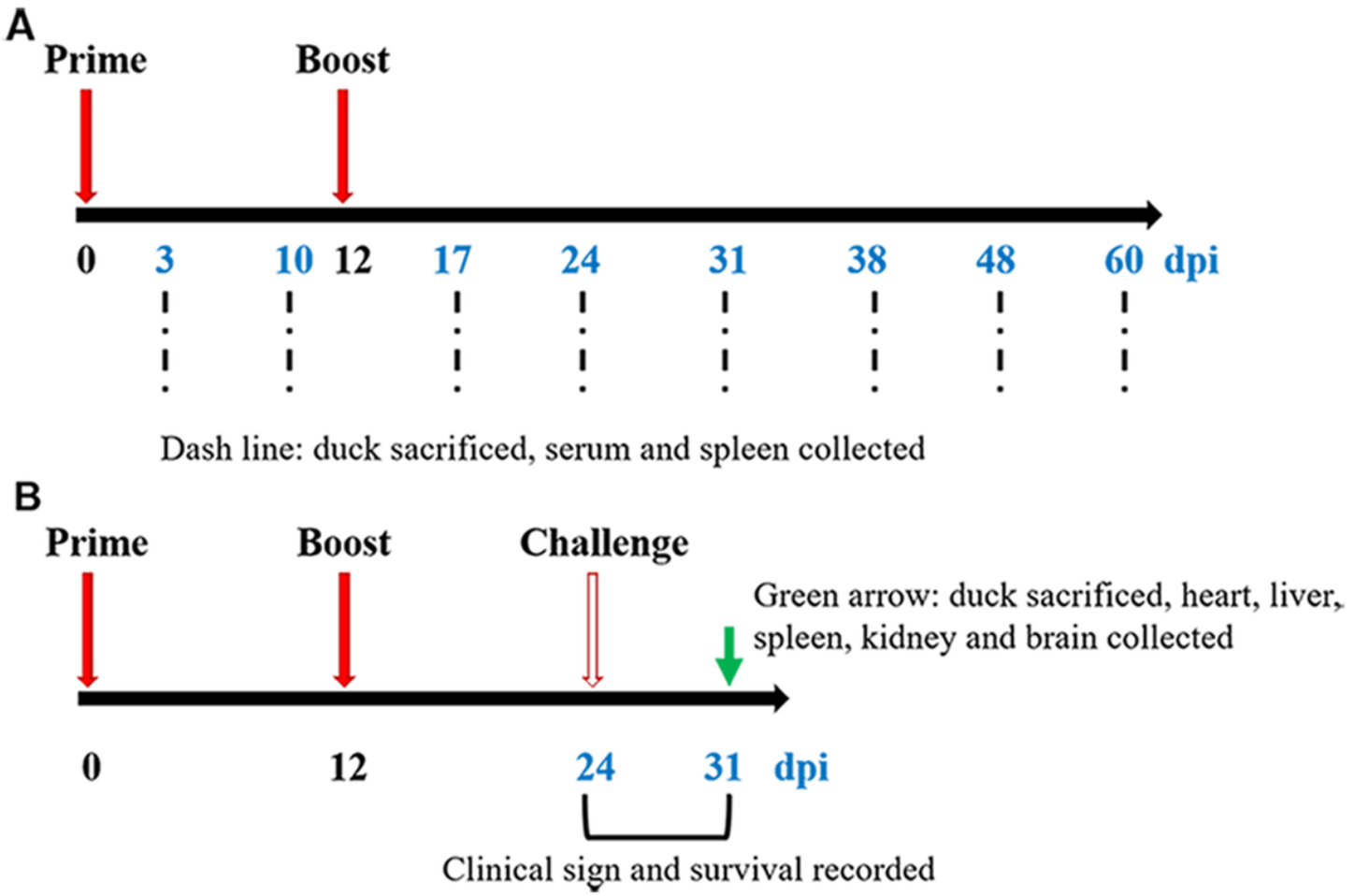

Figure 1

Duck experimental workflow. Duck experimental workflow. (A) Schedule of vaccination and sample collection. Ducks were vaccinated at 7-day-old and 19-day-old, respectively. Animals were sacrificed at 3, 10, 17, 24, 31, 38, 48 and 60 dpi (n=3 of each time point); (B) Schedule of challenge experiment. The ducks (n=10) of five groups were randomly selected at 12 days after the second immunization and challenged with 1ml 105.1 ELD50 TMUV to assay for the immune protection. The clinical signs and mortality were recorded for continuous 7 days after challenging.

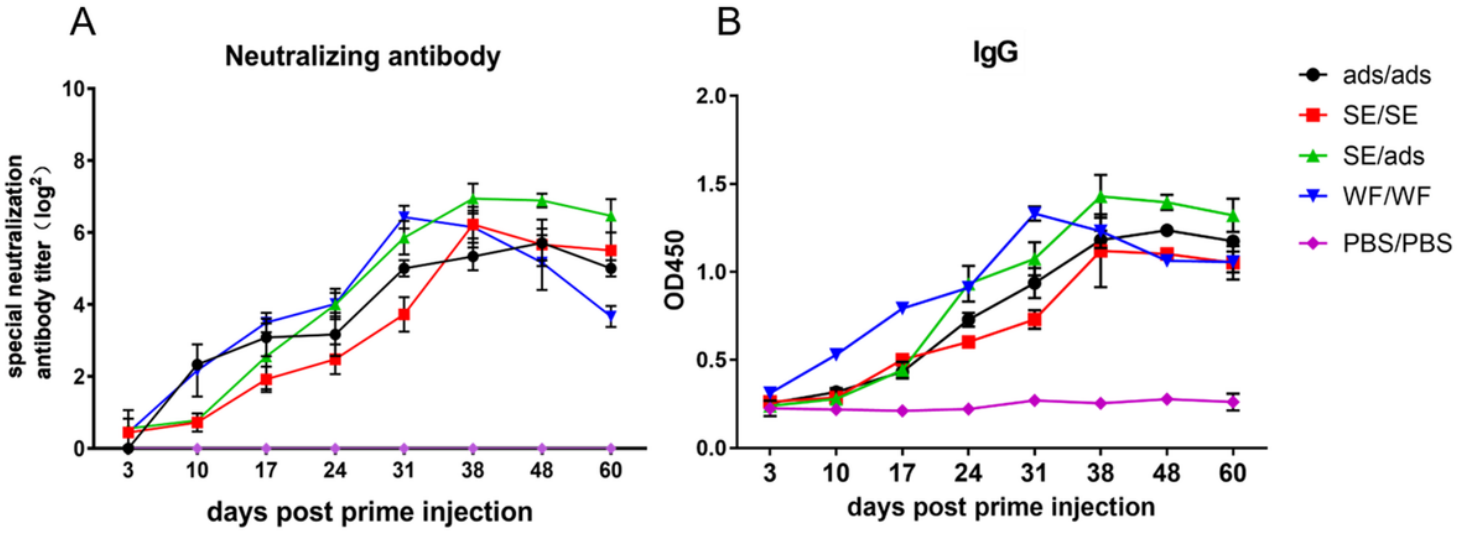

Figure 2

Production of specific antibodies. Production of specific antibodies. (A) The serum was collected from the ducks at 3, 10, 17, 24, 31, 38, 48, 60 dpi. Gradient dilution serum and viral fluids were mixed and incubated in the DEF cells for 1h, then removed the mixture and added DMEM with 2% serum, the DEF cells

were cultured at 37°C and observed for 5 days to record the lesions of cells. (B) The IgG antibodies in sera specific to TMUV E protein were checked by ELISA. The serum samples collected from ducks were diluted and incubated with the E protein coated plate. The specific anti-TMUV-E protein antibodies were measured by horseradish peroxidase-conjugated goat anti-duck IgG. The levels of IgG were shown as the means ± standard deviations (n=3 of each time point).

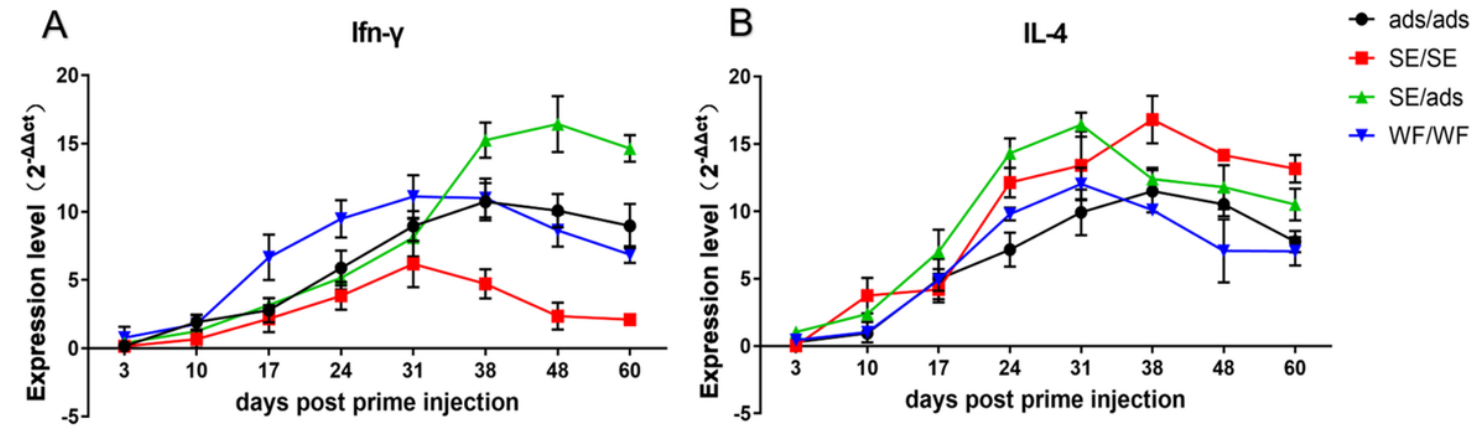

Figure 3

Comparative analysis the expression level of IL-4 and IFN-γ of the immunized ducks. Comparative analysis the expression level of IFN-γ and IL-4 of the immunized ducks. The samples were collected at 3, 10, 17, 24, 31, 38, 48 and 60 dpi to analyze the changes of cytokines by quantitative RT-PCR.

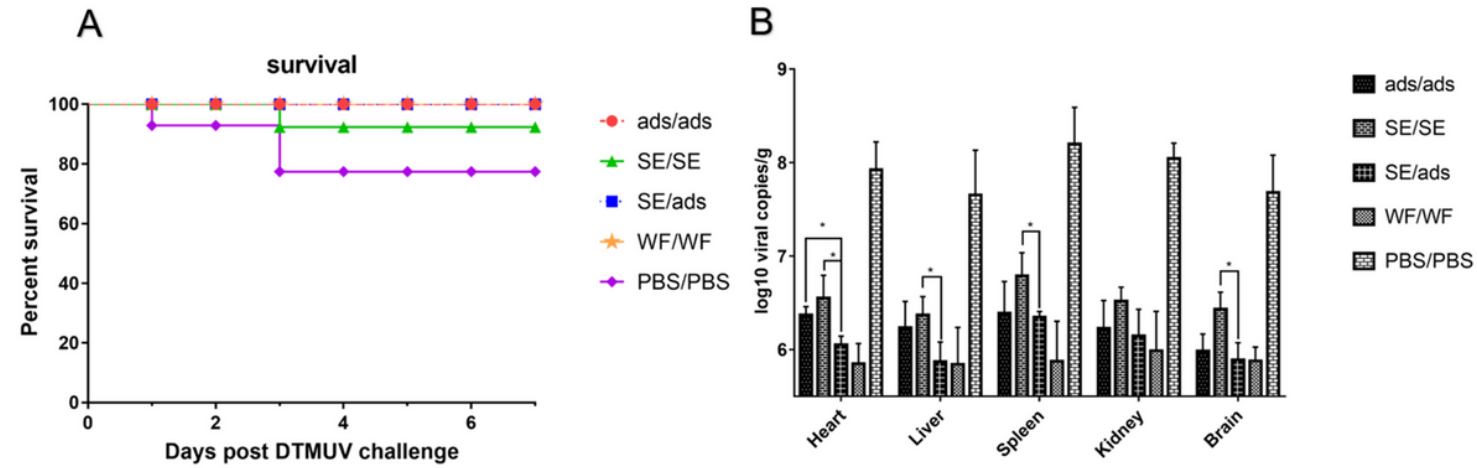

Figure 4

The protection of vaccines against TMUV challenge. The protection of vaccines against TMUV challenge. (A) Survival curves post challenge with TMUV. The immunized ducks (n=10 per group) were challenged with 1ml 105.1 ELD50 TMUV at 12 days after the second immunization. The survival was recorded for consecutive 7 days after challenge and graphed by Graphpad Prism v7.0. (B) Viral loads in tissues from ducks after challenge. Viral loads of tissues (heart, liver, spleen, kidney and brain) from each group were measured by quantitative RT-PCR. Data were expressed as mean ± SD (n=3). '*' presented a significant difference at P <0.05.

Figure 5

Microscopic lesions of the tissues after challenging with TMUV. Microscopic lesions of the tissues after challenging with TMUV (400×). There were no microscopic lesions in SE/ads and WF/WF groups, slight lesions in ads/ads and SE/SE groups, but the most severe lesions in PBS/PBS group.