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Virology

A recombinant adenovirus expressing the E protein of duck Tembusu virus induces protective immunity in duck

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ABSTRACT. Duck Tembusu virus disease, caused by the duck Tembusu virus (DTMUV), can lead to a severe reduction in egg production and growth retardation in laying ducks and ducklings, respectively. In this study, we engineered a novel recombinant adenovirus expressing the E protein of DTMUV (rAd-E) in AAV-293 cells (analyzed by western blot and indirect immunofluorescence assays). Intramuscular immunization of Cherry Valley ducks with rAd-E was performed to evaluate host cellular and humoral immune responses. Compared to the phosphate-buffered saline administered group and the negative control wild-type adenovirus (wtAd) group, the rAd-E vaccinated group showed increased cellular and humoral responses. The results from the cytokine release and lymphocyte proliferation assays showed that rAd-E induced a stronger cellular immune response than the control group ($P<0.01$), 4 weeks after primary immunization. The results of enzyme-linked immunosorbent and virus neutralization assays showed that rAd-E induced higher titers of specific neutralizing antibodies, 2 weeks after primary immunization. The DTMUV challenge experiment showed a higher survival rate (80%) of ducks in the rAd-E group, when challenged with 0.5 m*l* ($ELD_{50}=10^{-2.67}$/0.2 m*l*) of the DTMUV strain AH-F10. These results indicate that rAd-E effectively protects ducks against DTMUV infection. Therefore, rAd-E could be a vaccine candidate to provide an effective and safe method for prevention and control of DTMUV infection.

KEY WORDS: duck Tembusu virus, E protein, experimental infection, protection, recombinant adenovirus

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Duck Tembusu virus infection is a newly emerging infectious disease caused by the duck Tembusu virus (DTMUV); genus *Flavivirus*; family Flaviviridae. Until recently, egg-laying chickens [14], geese [6], pigeons [15], sparrows [21], and mosquitoes [9, 20] were the only known hosts of DTMUV in China. To avoid the propagation of DTMUV, ducks have become a major target animal group.

DTMUV is a small, enveloped, positive-stranded RNA virus. Mature virions have a diameter of 45–50 nm and contain three structural proteins, designated C (nucleocapsid), E (envelope), and prM (pre-membrane), as well as seven nonstructural proteins [11, 15]. Among these proteins, protein E is a major antigenic determinant and plays a dual role in host cell entry—attachment to cellular receptors and membrane fusion [16, 26, 29].

At present, there are no specific treatments or licensed vaccines available for protection against this pathogenic virus. Therefore, there is an urgent need for the development of an effective vaccine to prevent DTMUV infection. Recently, several approaches have been investigated to develop a DTMUV vaccine, including the use of inactivated viruses [12, 28], live-attenuated viruses [10, 19], subunit vaccines [16, 29], recombinant duck enteritis virus expressing E protein [1], and naked DNA [5]. With regard to the immunogenicity, efficacy, cost, and safety, adenoviruses have shown incredible promise, as vectors for recombinant vaccine development [7, 22]. Besides being safe, adenoviruses have the capacity to effectively induce both- humoral and cellular immune responses [4, 13].

Here, we report that immunization with a recombinant adenovirus expressing the E protein of DTMUV can effectively elicit neutralizing antibody production and prevents DTMUV infection without the need to immunize any virus particle.

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MATERIALS AND METHODS

Viral reference strain, materials, and animals

DTMUV strain AH-F10 and AAV-293 cells (higher viral titer-producing cells, derived from HEK 293) were provided by the Anhui Province Key Laboratory of Veterinary Pathobiology and Disease Control AH-F10 (median lethal dose for chicken embryos [ELD_{50}]=$10^{-2.67}$/0.2 m*l*, median tissue culture infective dose [$TCID_{50}$]=$10^{-6.5}$/0.1 m*l*). Rabbit anti-E polyclonal antibody was prepared in our laboratory [24]. The RAPAD Adenovirus Expression System was purchased from Cell Bio Labs (U.S.A.). The expression system includes shuttle vector pacAd-CMV K-NpA and backbone vector pac—devoid of the left-hand ITR—Ad5 9.2–100, the packaging signal and E1 sequences. Horseradish peroxidase (HRP)-conjugated and FITC-conjugated goat anti-rabbit antibodies were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). HRP-conjugated goat anti-duck antibody was purchased from KPL (U.S.A.). The RNA extraction kit and restriction enzymes *Eco*RI and *Bam*HI were purchased from New England Biolabs (Ipswich, MA, U.S.A.). TRIzol reagent was purchased from Tiangen Biotech (Beijing) Co., Ltd. (Beijing, China). Duck interleukin (IL)-4 and interferon (IFN)-γ enzyme-linked Immunosorbent assay (ELISA) kits were purchased from Calvin Biotechnology Co., Ltd. (Suzhou, China).

Nine-day-old specific pathogen-free (SPF) embryonated chicken eggs were purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China). Unimmunized seven-day-old cherry valley ducks were purchased from a commercial hatchery, with a controlled sanitary status, from Wuxi, China.

We confirmed that all animal experiments were carried out accordance with the guideline of the Guide for the Care and Use of Laboratory Animal of the Institutional Animal Care and Use Committee (IACUC) set by Anhui Agricultural University. All animal experiments were approved by the Animal Care and Use Committee of Anhui Agricultural University (Approval number AHAU2015-23).

Construction of the recombinant adenoviruses rAd-E

Based on the E gene sequence of DTMUV strain AH-F10 (GenBank accession number KM102539), a primer pair was designed and synthesized by Qingke Biological Technology Co., Ltd. (Shanghai, China) with the following sequences: forward 5′- GGA ATT CGC CAC CAT GTT CAG CTG TCT GGG GAT GC-3′ containing a *Eco*RI site (underlined) and reverse 5′- CGG GAT CCG GCA TTG ACA TTT ACT GCC AG-3′ containing a *Bam*HI site (underlined).

Viral RNA, extracted with TRIzol, was used to synthesize template cDNA, according to the manufacturer's protocol. The target E gene (1,515 bp) was amplified by PCR, using appropriate primers and template (94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 49°C for 30 sec, 72°C for 90 sec, and a final extension at 72°C for 10 min).

The E gene was digested with *Eco*RI and *Bam*HI, and cloned into the shuttle vector pacAd5 CMV K-N pA. DNA sequencing was used to verify pacAd5-E (Sanggong Biotechnology, Sanggong, China). The recombinant vector pacAd5-E and backbone vector pac Ad5 9.2–100 were co-transfected into AAV293 cells, to produce an adenovirus recombinant E protein (named rAd-E), according to the manufacturer's protocol. A wild-type adenovirus (wtAd) served as negative control. rAd-E was propagated in AAV-293 cells and purified via plaque test three times. The genetic stability of the viruses was determined by PCR. The virus titer $TCID_{50}$ was determined from the 8th generation of rAd-E, according to Reed-Muench method.

Immunofluorescence analysis (IFA)

The rAd-E was propagated in AAV-293 cells. After removing the supernatant, the cells were fixed with 4% paraformaldehyde for 15 min. The cells were washed thrice, incubated with rabbit anti-E polyclonal serum (at a dilution of 1:2,000) in a humidified chamber, and subsequently stained with FITC-conjugated goat anti-rabbit IgG. After washing in phosphate-buffered saline (PBS), the specific fluorescence of the infected cells was visualized using a fluorescence microscope (Olympus Corp., Tokyo, Japan).

Western blot analysis

For protein expression analysis, lysates from rAd-E-transduced cells were electrophoresed on a 10% SDS-PAGE and the proteins transferred onto a nitrocellulose membrane by electroblotting (Bio-Rad Laboratories, Hercules, CA, U.S.A.). wtAd was used as a negative control. Western blot analysis was performed using standard methods with a primary rabbit anti-E polyclonal antibody (dilution, 1:500) and a secondary HRP-labeled goat anti-rabbit IgG antibody (dilution, 1:5,000).

Immunization experiments and viral challenge

Thirty seven-day-old Cherry Valley ducks were randomly divided into three groups (named rAd-E, wtAd, and PBS) of 10 ducks each. All ducks were confirmed to be free of DTMUV infection (titer=0), by detection of the neutralizing antibodies prior to inoculation. To improve the concentration of antigen (E protein) and reduce the immunization dosage, the recombinant rAd-E and wtAd were kept at a high concentration of 1:10. The ducks in the rAd-E and wtAd groups were immunized twice, intramuscularly, at 2-week intervals with 10^8 $TCID_{50}$ of either rAd-E or wtAd, respectively. Group PBS (negative control) was inoculated subcutaneously with 500 μl of PBS only. Serum samples were collected at 0, 1, 2, 3, and 4 weeks post- primary immunization. Four weeks after the primary immunization, all experimental groups were injected intramuscularly with 0.5 m*l* of DTMUV strain AH-F10 (ELD_{50}=$10^{-2.67}$/0.2 m*l*). The ducklings were monitored for 14 days and all instances of death were recorded.

Cytokine release assay and lymphocyte proliferation assay

The cytokine levels of each experimental group were determined in the serum, collected after 0, 1, 2, 3, and 4 weeks of immunization, using commercial IL-4 and IFN-γ ELISA kits according to the manufacturer's protocol.

Lymphocyte proliferation assay was conducted 4 weeks after primary immunization. Briefly, peripheral blood mononuclear cells (PBMCs) were collected from three ducks, in each experimental group, and then separated by Ficoll-Hypaque density gradient centrifugation [8]. PBMCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% heat-inactivated FCS. Cells (1×10^5) were plated in 96-well plates (in triplicate) and incubated with lymphocyte mitogen ConA at a concentration of 5 μg/m*l*, for 44 hr. Subsequently, the absorbance (OD_{490}) was measured. An equal volume of RPMI 1640 medium, without any cells, served as a blank control. The stimulation index was calculated as the ratio of the average OD_{490} value of stimulated cells to that of the negative control.

Specific antibody detection

Indirect ELISA was performed to determine the antibody titers in sera from each experimental group, after 0, 1, 2, 3, and 4 weeks after primary immunization. Briefly, ELISA plates were coated with the purified DTMUV E protein (100 μ*l* at 2.96 μg/m*l*; at 4°C). The plates were blocked; serum samples were added at a dilution of 1:800. HRP-conjugated goat anti-duck IgG was used to detect bound antibodies and finally, the OD at 450 nm was measured. The results are presented as the mean value ± SD.

Virus neutralization assay (VNA)

Serum samples were collected at weeks 0 (pre-immunization), 2, and 4 after primary immunization for the detection of DTMUV-specific VNA titers. Briefly, the serum samples were incubated at 56°C for 1 hr to inactivate complement molecules. Sera of distinct groups were serially diluted (two-fold), mixed with an equivalent volume of 200 ELD_{50} of DTMUV strain AH-F10 (in a 100-μ*l* volume), and incubated for 1 hr at 37°C. These virus-serum mixtures were injected into the allantoic cavity of 9-day-old SPF embryonated chicken eggs. After incubation at 37°C for 5–7 days, we determined the highest serum dilution that inhibited DTMUV infection or death of more than 50% of the SPF chicken embryos, and serum neutralizing antibody titer was calculated. Antibody titers were calculated using the Reed and Muench method [18]. Unimmunized serum was used as a negative control.

Statistical analysis

All assays were performed in triplicates, at least. All the data were graphed and analyzed for statistics using Prism 5 software (GraphPad Software Inc., La Jolla, CA, U.S.A.). The means of two groups were compared with the two-tailed Student's *t*-test, whereas multiple comparisons were carried out by one-way analysis of variance. All probability values of $P<0.05$ were considered statistically significant.

RESULTS

Construction of recombinant adenoviruses rAdE

A 1.5-kb DNA fragment (E gene) was amplified by reverse transcription-PCR from the genome of DTMUV strain AH-10 and cloned into the pacAd5 CMV K-N pA shuttle vector. DNA sequencing revealed that the E gene sequence in the recombinant plasmids was correct. A recombinant adenovirus, designated as rAd-E, was engineered and propagated in AAV-293 cells. Typical cytopathic effects, characterized by rounding and detachment, were observed 5 days post-transfection, but not in the negative control cells treated with PBS. The recombinant adenoviruses were verified by PCR. The rAdE strain propagated well in AAV-293 cells with a titer of $10^{-6.17}$/0.1 m*l*.

Validation of protein expression in vitro

Expression of the DTMUV E protein, through rAd-E in cell culture, was confirmed using western blotting and IFA. As shown in Fig. 1A, a specific band of approximately 55 kDa (consistent with the predicted size) was observed from the lysates of rAd-E infected-AAV-293 cells. No protein band was detected in lysates from wtAd-infected cells. AAV-293 cells inoculated with rAd-E were fixed with cold acetone and treated with specific polyclonal anti-E antibody. DTMUV-specific fluorescence appeared in the infected cells only, while the negative control displayed no specific fluorescence (Fig. 1B). The results of western blotting and IFA indicate that the rAd-E can express the target proteins *in vitro*.

Cellular immune response in ducks immunized with rAd-E

To further evaluate cellular immunity responses induced by rAd-E, a cytokine release assay (IFN-γ and IL-4) was performed. As shown in Fig. 2A and 2B, the levels of IFN-γ and IL-4 in the ducks vaccinated with rAd-E were statistically higher than the two control groups, 4 weeks ($P<0.01$) after primary immunization.

A lymphocyte proliferation assay was used to investigate the cell-mediated immune response in ducks, induced by rAd-E, 4 weeks after primary immunization. As shown in Fig. 2C, the rAd-E groups showed more vigorous lymphocyte response than the two control groups, 4 weeks ($P<0.01$) after primary immunization. These results indicate that rAd-E displays obvious and intense cellular immune responses in ducks.

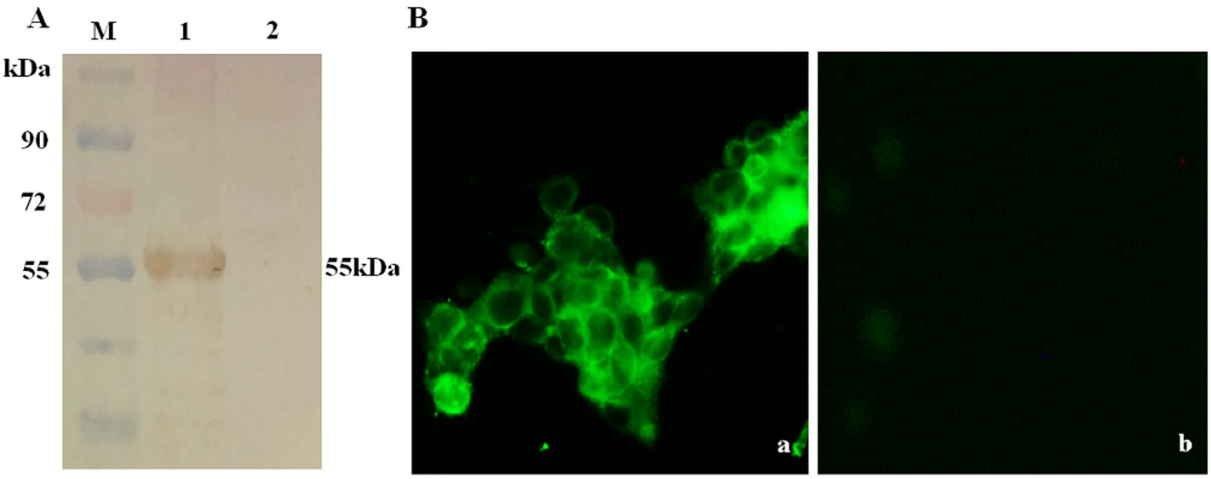

Fig. 1. Identification of the proteins expressed by recombinant adenoviruses. Western blotting analysis of lysates from cells infected with recombinant adenoviruses, with rabbit anti-E serum. Lane M, protein standard marker; lane 1, AAV-293 cells infected with rAd- E; lane 2, AAV-293 cells infected with wtAd, negative control. (B) IFA analysis of E protein expression in AAV-293 cells infected with rAd-E. After 48 hr, the infected cells were detected with rabbit anti-E serum, followed by FITC-conjugated goat anti-rabbit IgG and observed under a fluorescence microscope. (a) AAV-293 cells infected with rAd-E; (b) AAV-293 cells infected with wtAd, negative control.

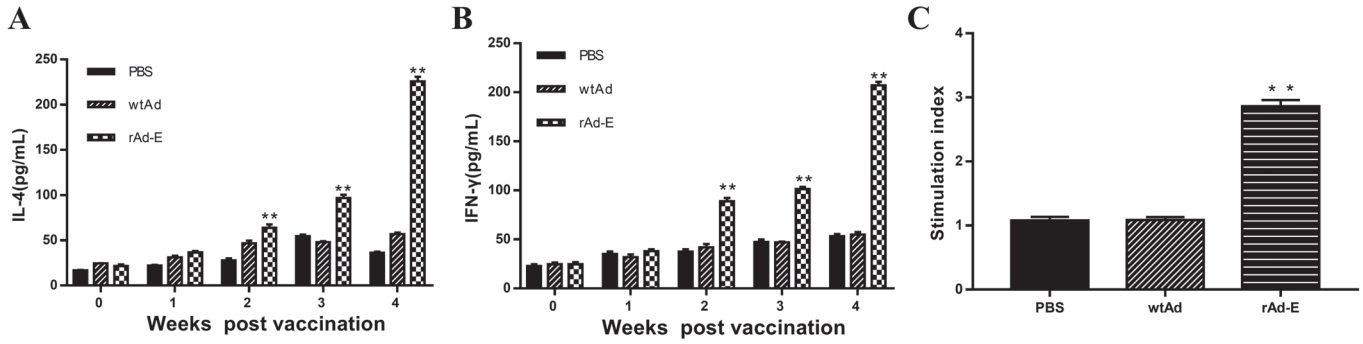

Fig. 2. Estimation of cytokine levels and lymphocyte proliferation following immunization in ducks vaccinated with rAd-E, wtAd, or PBS. Serum samples were isolated at 0, 1, 2, 3, and 4 weeks after primary immunization for cytokine analysis using ELISA kits for (A) IFN-γ and (B) IL-4. (C) Four weeks after primary immunization, proliferative responses of peripheral blood lymphocytes were evaluated with a lymphocyte proliferation assay. Data are presented as the mean concentration (*p*g/m*l*) ± SD. The SD values are shown as error bars. All experiments were repeated more than three times. ***P*<0.01.

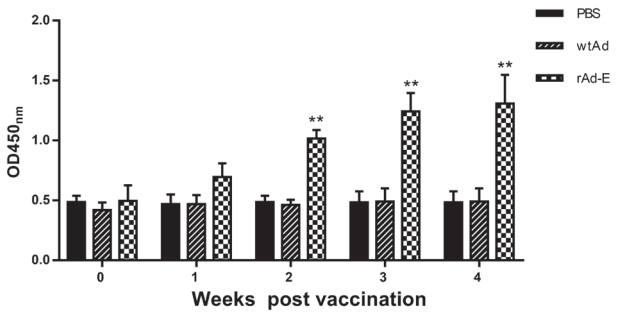

Fig. 3. Antibody estimation following immunization in ducks vaccinated with rAd-E, wtAd, or PBS. ELISAs were conducted to determine the antibody titers in serum samples (n=5) that were collected at various time-points. The results are presented as the mean ± SD of A_{450}. The SD values are shown as error bars. All experiments were repeated more than three times. ***P*<0.01.

Table 1. Neutralizing antibody titers of ducks (results are shown as mean ± SD)

Groups	Week 2	Week 4
PBS	0	0
wtAd	0	0
rAd-E	3.82 ± 0.36	6.51 ± 0.83

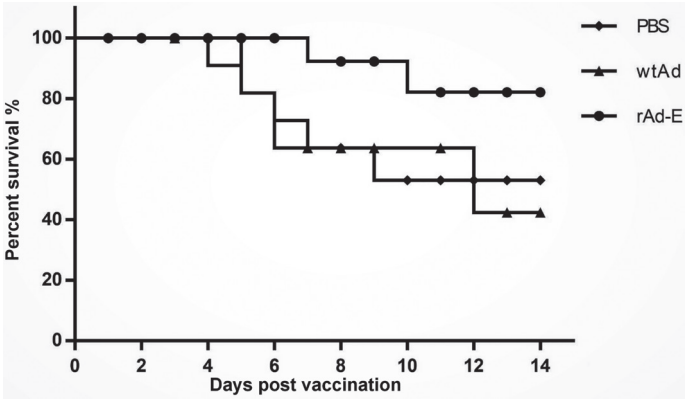

Fig. 4. Survival curves of ducks (n=10) vaccinated with rAd-E, wtAd, or PBS. The statistical significance of differences in mortality between groups was determined using the Kaplan–Meier method, and analyzed with a Log-rank (Mantel-Cox) test. For rAd-E vs wtAd, $P<0.05$; and for rAd-E vs PBS, $P<0.05$.

Humoral immune responses in ducks immunized with rAd-E

To determine whether rAd-E can induce DTMUV-specific immune response in ducks, serum samples−collected at 0, 1, 2, 3, and 4 weeks after primary immunization−were used to calculate the presence of E-specific neutralization antibodies. As shown in Fig. 3, E-specific antibodies were detected, 1 week after primary immunization, in all the ducks immunized with group rAd-E. After two immunizations, the mean antibody level increased more rapidly in the group rAd-E, with statistically significant differences, compared to wtAd and PBS control groups ($P<0.01$).

As shown in Table 1, all ducks immunized with rAd-E developed neutralizing antibodies 2 weeks after the primary immunization, and the titers increased up to 6.51 ± 0.83, 4 weeks after the primary immunization. None of the ducks in the wtAd and PBS control groups produced detectable neutralizing antibodies during the experiment. This finding indicated that the recombinant adenovirus expressing the E protein enhanced the humoral immune response.

Protection of ducks against DTMUV challenge

To investigate whether a higher immune response was correlated to increased protection, immunized ducks were challenged (4 weeks after primary immunization) with a dose of 0.5 m*l* ($ELD_{50}=10^{-2.67}$/0.2 m*l*) DTMUV strain AH-F10. Subsequently, all ducks were housed in isolation and monitored for 14 days. In the control group, the ducks showed serious weight loss and typical symptoms, including inappetence, keratitis, tearing, ataxia, tilted head, and paralysis; while very slight signs of illness were observed in ducks immunized with rAd-E. As shown in Fig. 4, control ducklings and the ducklings immunized with rAd-E had an overall survival rate <50 and 80%, respectively.

DISCUSSION

The antigen delivery pathway is a key inducer of protective immune response by vaccines against pathogens [17]. An advantage of the adenoviral vector system is the ability to generate high-titer recombinant adenovirus, delivery of the exogenous gene to actively dividing cells, and the production of a replication-incompetent virus, thus, enhancing the biosafety of the system [2, 3, 27]. To date, many recombinant adenovirus-expressed proteins of infectious agents have proven effective, as vaccines [4, 13, 25]. In this study, the E protein of DTMUV was expressed in a recombinant adenovirus. The rAd-E inoculated in AA293 cells could be successfully propagated at a high titer of $10^{-6.17}$/0.1 m*l*.

Humoral immunity primarily produces neutralizing antibodies, with protective effects that can eliminate the virus infectivity. Neutralizing antibodies can bind to antigenic determinants on viral surface and disable the virus from adsorbing and transmitting, to prevent the occurrence and spread of infection.

The immunogenicity and protective efficacy of the rAd-E was evaluated in a duck model. After two immunizations, the mean antibody level increased rapidly in the group rAd-E, as compared, the wtAd and PBS control groups ($P<0.01$). The average neutralizing antibody titer in the ducks vaccinated with rAd-E increased from 3.82 ± 0.36 at 2 weeks to 6.51 ± 0.83 at 4 weeks after primary immunization. This finding indicates that rAd-E could stimulate the production of antibodies against DTMUV infection in ducklings.

Although, the favored belief dictates that protective immunity to DTMUV prevails due to neutralizing antibodies, a T-cell response is also indispensable for effective immunity. IL-4 plays a critical role in regulating the behavior of hematopoietic cells. In T-cells, IL-4 acts as a co-stimulant of cell growth and controls Th2 polarization. IFN-γ is a key cytokine produced primarily by T cells and natural killer-cells, which can facilitate the host defense against intracellular pathogens. In this study, both antibodies and

cytokines were measured to evaluate the immune effect of rAd-E. After two immunizations, the levels of DTMUV-specific, IL-4 and IFN-γ stimulated-lymphocyte proliferation by rAd-E were significantly higher than those of the wtAd group ($P<0.01$). These results suggested that rAd-E could stimulate a strong T cell response against DTMUV. Stimulation of both cellular and humoral immune responses against DTMUV could strengthen the protection of rAd-E against DTMUV.

Li Guoxin [10] reported that a live attenuated vaccine poses low safety risks to mammals. Wang Hong jiang [23] used the genetic backbone of JEV vaccine strain SA14-14-2, which has residual neurovirulence in mice, hindered further development of ChinDTMUV as a vaccine candidate. Compared to the PBS and wtAd groups, rAd-E immunized group showed markedly lower mortality rate, indicating that the effect of adenovirus recombinant vector should not be ignored. The E protein of DTMUV is critical for the induction of VNA and protective immunity. Thus, the E protein is related to protection of animals against virulent DTMUV. The results of the present study showed that the survival rate of the rAd-E group was 80%, which is still less than 100%. We suspect that the reduced protective immune responses were due to the absence of antigens (capsid, prM), which are typically present in the inactivated or live-attenuated vaccine. Additionally, the oral route or higher immunization doses of rAd-E should be considered in future animal experiments.

These data reveal that a recombinant adenoviral vector could be used as a potential antigen delivery system in the development of DTMUV vaccine. The generated rAd-E was highly immunogenic and evoked strong and sustained systemic antibody response in ducks. However, it was not found to statistically correlate with DTMUV disease survival rates. Therefore, the impact of a higher dose of rAd-E (viral particles per animal) and an oral delivery route needs to be further investigated in clinical trials.

CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

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