

<https://doi.org/10.1038/s41541-025-01146-5>

An mRNA vaccine provides effective protection against Duck Tembusu Virus infection

Check for updates

Shi Xu^{1,6}, Lijiao Zhang^{2,3,4,6}, Caiyi Fei¹, Guilai Liu¹, Mengwei Xu¹, Rui Liu¹, Jing Li¹, Aili Wang¹, Qing He¹, Ke Cai⁵, Qingtao Liu^{2,3,4} ✉ & Tiyun Han¹ ✉

As an emerging flavivirus, Duck Tembusu virus (DTMUV) causes severe neurological disorder and acute egg drop syndrome in poultry. Herein, we report the development of a highly effective mRNA vaccine against DTMUV using lipid nanoparticle (LNP) delivery technology. We engineered an mRNA construct encoding the pre-membrane (prM) and envelope (E) proteins of DTMUV, with an upstream signal peptide to enhance secretion. Two doses of our mRNA vaccine elicited robust neutralizing antibody titers and conferred 100% protection against challenge with a prevalent virulent DTMUV strain. Notably, in a head-to-head comparison, the neutralizing antibody titers induced by our mRNA vaccine were approximately 40-fold of a commercial live-attenuated vaccine (FX2010-180P). Importantly, our mRNA vaccine demonstrated efficient maternal antibody transfer via egg yolk deposition, indicating the potential for protecting offspring through maternal immunity. These findings demonstrate the superiority of our mRNA vaccine platform, as well as the feasibility of applying mRNA vaccines in poultry.

Flaviviruses pose significant threats to both human health and animal health. Duck Tembusu virus (DTMUV) is a member of *Ntaya* virus group within the *Flaviviridae* family. DTMUV leads to a range of clinical symptoms, including retarded growth, diarrhea, and severe neurological disorders¹. Therefore, DTMUV has become a major concern for the poultry industry, particularly in Asia. Since 2010, DTMUV has spread rapidly in China, causing notable economic losses due to high mortality rates in ducklings and egg drop syndrome in ducks^{2,3}.

The economic impact of DTMUV outbreaks has driven intensive research into vaccine development. Current preventive strategies primarily rely on inactivated and live-attenuated vaccines⁴. However, these traditional vaccines have certain shortcomings. Inactivated vaccines often require adjuvants and multiple rounds of immunizations to elicit sufficient immune responses, leading to increased production costs and potential adverse reactions⁵. They primarily induce humoral immune response, potentially leaving gaps in cellular immune response⁶. Live-attenuated vaccines are capable of stimulating both humoral and cellular immune responses with low doses; however, they carry the risk of recombination with prevalent strains and consequent virulence increase. Therefore, live-attenuated

vaccines always bring significant concerns in immunocompromised individuals or in areas with sizeable wild bird populations as wild strain reservoirs^{7,8}. Additionally, differentiating infected from vaccinated animals is challenging with the application of live-attenuated vaccines, complicating disease surveillance and control efforts^{8–10}. Moreover, the residual virulence of live-attenuated DTMUV vaccines often exhibit clinical adverse reactions that significantly impact the economic viability of duck farming¹¹.

Both inactivated and live-attenuated vaccines are struggling with rapid mutation and immune escape of viral strains, especially for RNA viruses like DTMUV that have higher mutation rates than DNA viruses¹². In light of these challenges, there is an urgent need for innovative vaccine technologies that can address these shortcomings and offer improved efficacy, safety, and adaptability. Therefore, Messenger RNA (mRNA) vaccines have emerged as a promising platform with satisfactory solutions¹³. The modular nature of mRNA vaccine design enables rapid adaptation of successful strategies from related viruses. The success of mRNA vaccines in combating the SARS-CoV-2 pandemic has demonstrated the capability of mRNA technology in addressing urgent public health challenges¹⁴, accelerating mRNA vaccine research on other viral diseases.

¹Nanjing Chengshi (TheraRNA) Biomedical Technology Co. Ltd., Nanjing, China. ²Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture; Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base of Ministry of Science and Technology, Nanjing, China. ³Jiangsu Coinnovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou, China. ⁴GuoTai (Taizhou) Center of Technology Innovation for Veterinary Biologics, Taizhou, China. ⁵Xuzhou Liyuan Veterinary Service Co. Ltd., Xuzhou, China. ⁶These authors contributed equally: Shi Xu, Lijiao Zhang.

✉ e-mail: taoqingliu2013@163.com; hantiyun@therarna.cn

In this study, we developed an mRNA vaccine against DTMUV, focusing on its capability of inducing substantial neutralizing antibodies and protection against viral challenge. We also explored the potential for maternal antibody protection by determining the neutralizing antibodies in the egg yolks of vaccinated ducks. This research not only addresses the specific challenge of DTMUV, but also provide the first feasibility study of mRNA vaccine in poultry. By leveraging the modular nature of mRNA vaccines and maternal antibody protection, we aim to pave the way for more rapid, effective, and safe vaccine development against unmet medical needs in animal health.

Results

Design of DTMUV prM/E construct and viral protein expression

Inspired by vaccine designs of other flaviviruses, particularly Zika and dengue viruses, we designed a fusion antigen encoding the pre-membrane (prM) and envelope (E) structural proteins of DTMUV to recapitulate the virion architecture^{15,16}. The mRNA design incorporates optimized 5' and 3' untranslated regions, a type 1 cap structure, and the signal peptide from duck CD5 to enhance expression and secretion (Fig. 1A). The naked mRNA was transfected into 293 T cells using LNP3 to confirm robust protein expression, as shown in western blot analysis (Fig. 1B). After verifying successful expression in vitro, the mRNA was then encapsulated within lipid nanoparticles (LNPs) to facilitate efficient cellular delivery and protein production in vivo.

Two doses of mRNA vaccines provided complete protection against DTMUV infection in SPF ducks

We immunized specific pathogen-free (SPF) ducks using a prime-boost regimen, with initial priming on Day 7 followed by a booster on Day 28 (Fig. 2A). Serological analysis revealed a rapid and robust induction of neutralizing antibodies, with titers reaching a peak by Day 42 (mean titer ~1:64) and maintaining high levels through Day 49 (Fig. 2B). This sustained neutralizing antibody levels demonstrated the potent immune response elicited by the mRNA vaccine. In contrast, the control animals exhibited no detectable neutralizing antibodies (titer < 1:4) throughout the study period. These findings underscored the vaccine's ability to elicit a durable and specific humoral response against DTMUV.

Challenge studies with the highly virulent DTMUV strain JS804 on Day 49 revealed complete protection in all ducks vaccinated with our mRNA vaccine (5/5), with no clinical manifestations of infection (Fig. 2C). Conversely, all control ducks (5/5) developed severe symptoms, including pyrexia (>41 °C), anorexia, and neurological complications. The observed protection correlated strongly with neutralizing antibody titers. These results not only demonstrate the vaccine's efficacy in preventing DTMUV-induced morbidity but also elucidate the critical role of humoral immune response in mediating protection against DTMUV.

DTMUV mRNA vaccine demonstrated superior efficacy over commercial live attenuated vaccine in male breeder drakes

We performed a head-to-head comparison study on our DTMUV mRNA vaccine and the commercial live attenuated vaccine (FX2010-180P) in male breeder drakes (Fig. 3A). By Day 28 post-initial immunization, the mRNA vaccine induced remarkably high levels of DTMUV-specific antibodies, with mean ELISA titers approximately 40-fold of those elicited by the commercial vaccine (3,875 ± 1,805 versus 98 ± 127, respectively; *****P* < 0.001) (Fig. 3B). This robust humoral response demonstrates the superior performance of our mRNA vaccine.

We also analyzed neutralizing antibody titers in a subset of four randomly selected drakes from each group. Consistent with the ELISA findings, the mRNA vaccine elicited substantially higher neutralizing antibody titers (mean titer 1:512, range 1:256–1:1024) compared to the commercial live-attenuated vaccine (mean titer 1:32, range 1:16–1:64) (Fig. 3C, D). This strong correlation between binding and neutralizing antibodies underscores the quality of the immune response elicited by our mRNA vaccine.

DTMUV mRNA vaccine elicits robust neutralizing antibody responses in laying ducks and efficient maternal antibody transfer

To evaluate mRNA vaccine's capability for inducing maternal antibody transfer and passive immune protection, we performed a study in laying ducks (Fig. 4A). Following the prime-boost immunization strategy, with doses administered on Days 0 and 14, we observed robust induction of DTMUV-specific neutralizing antibodies in laying ducks (Fig. 4B). Serological analysis

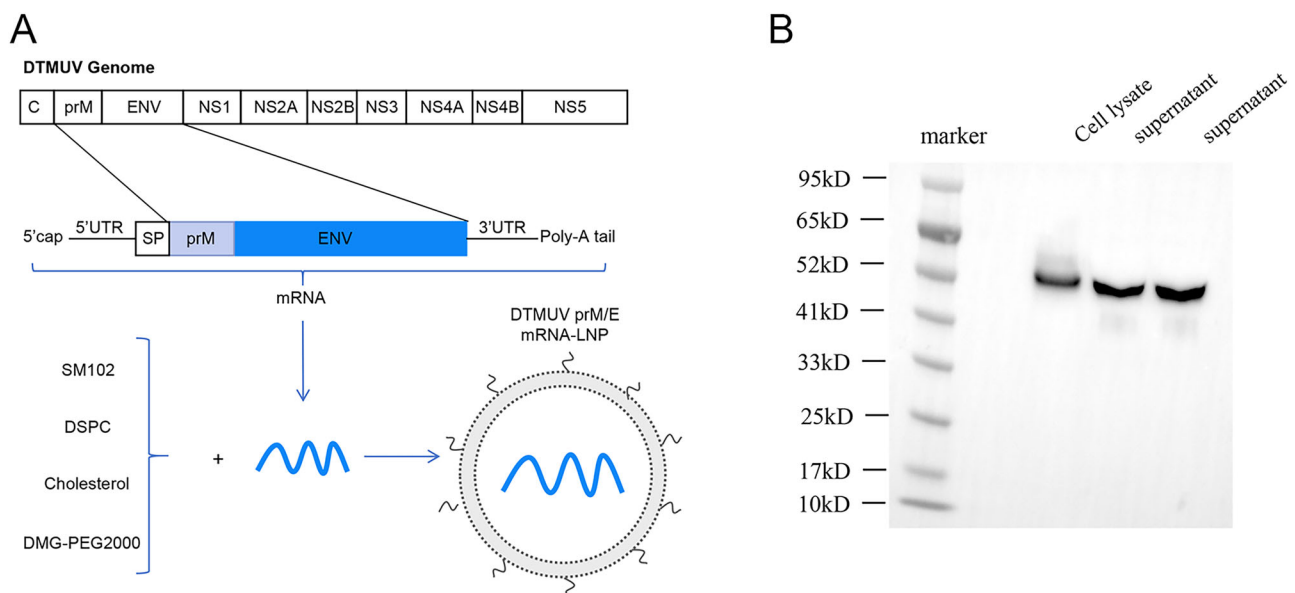


Fig. 1 | Design and characterization of DTMUV prM/E mRNA-LNP vaccine. **A** Schematic representation of mRNA vaccine design and LNP formulation. The full-length DTMUV genome encodes structural (C, prM, E) and non-structural (NS1-NS5) proteins (top). The engineered mRNA construct (middle) comprises optimized 5' and 3' UTRs, a signal peptide (SP), and the prM/E genes, terminated with a poly-A tail. The mRNA is encapsulated in lipid nanoparticles (bottom)

composed of ionizable lipid (SM102), helper lipids (DSPC, cholesterol), and PEG-modified lipid (DMG-PEG2000). **B** Western blot analysis of prM/E protein expression in transfected 293 T cells. Cell lysates and culture supernatants were analyzed using anti-E protein antibodies. The E protein (~54 kD) was detected in both cell lysate and supernatant, confirming successful expression and secretion. Molecular weight markers (in kD) are indicated on the left.

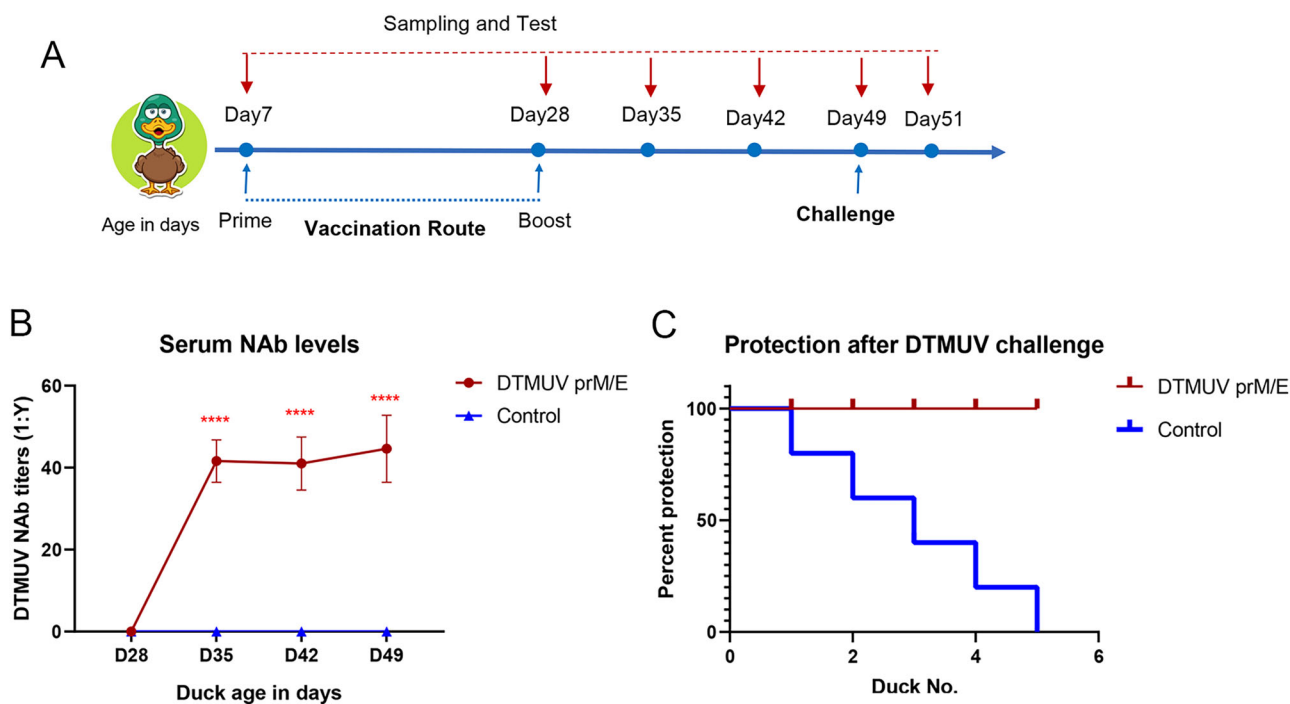


Fig. 2 | Protective efficacy of DTMOV mRNA vaccine in SPF ducks.

A Experimental timeline depicting the prime-boost vaccination schedule and subsequent viral challenge. SPF ducks received primary immunization on Day 7 followed by a boost dose on Day 28. Serum samples were collected at indicated timepoints (red arrows) for antibody analysis. Virus challenge was performed on Day 49 with DTMOV strain JS804. **B** Kinetics of DTMOV-specific neutralizing

antibody titers in duck sera following immunization. **C** Protection after DTMOV challenge in vaccinated and control ducks. Data points represent mean ± SEM ($n = 5$). Statistical analyses were performed using two-way ANOVA with Bonferroni's multiple comparisons test and log-rank (Mantel-Cox) test. Statistical significance between vaccinated and control groups is indicated by asterisks (**** $P < 0.0001$).

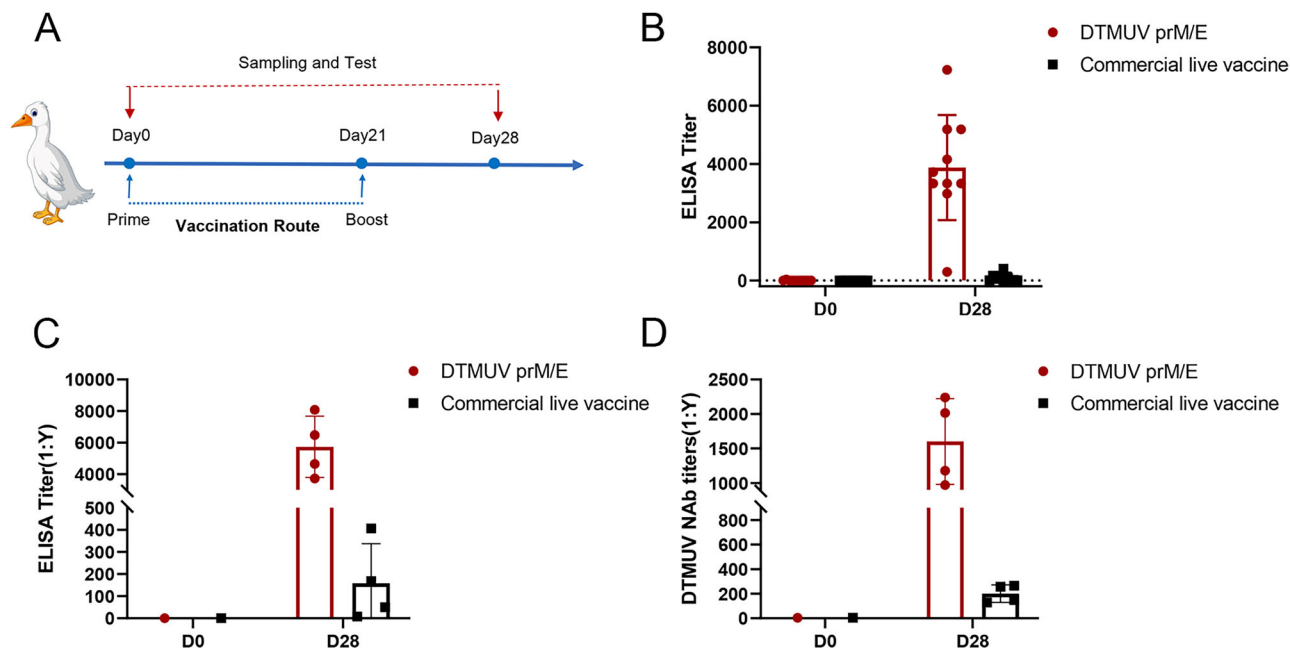


Fig. 3 | Comparative immunogenicity of DTMOV mRNA vaccine versus commercial live attenuated vaccine in male breeder drakes. **A** Schematic illustration of the prime-boost vaccination schedule and sampling timeline. **B** DTMOV-specific antibody titers measured by ELISA in all vaccinated drakes ($n = 10$ per group). Individual data points and mean ± SEM are shown. **C** DTMOV-specific antibody titers in the randomly selected four drakes for neutralization antibody test.

D DTMOV-specific neutralizing antibody titers in the randomly selected drakes ($n = 4$ per group) at Day 28 post-immunization. Individual titers and mean ± SEM are presented. For both ELISA and neutralization assays, samples were analyzed in technical duplicates. Statistical significance was determined using two-way ANOVA with Bonferroni's multiple comparisons test (**** $P < 0.0001$).

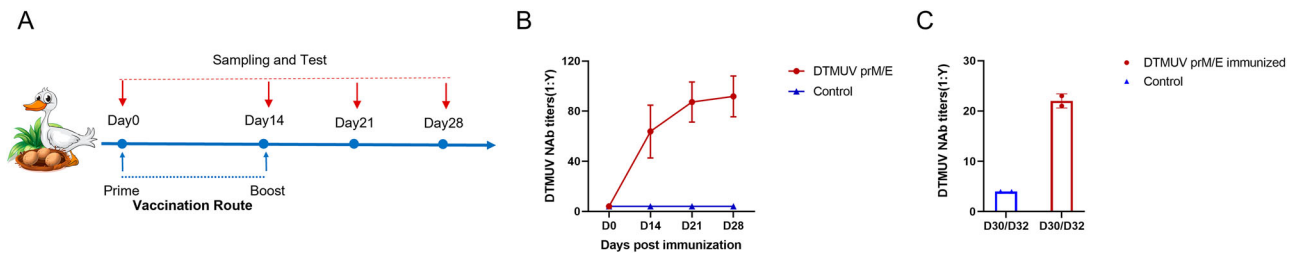


Fig. 4 | Immunization schedule and neutralizing antibody responses in vaccinated laying ducks. **A** Schematic representation of the prime-boost vaccination regimen and sampling timeline. Ducks received prime and boost immunization on Day 0 and Day 14, respectively. Serum samples were collected on Days 0, 14, 21, and

28 for antibody analysis. **B** Kinetics of DTMUV-specific neutralizing antibody titers in duck sera following immunization. Data points represent mean \pm SEM ($n = 6$). **C** DTMUV-specific neutralizing antibody titers in egg yolks collected between Days 30–32 post-primary immunization. Data are presented as mean \pm SEM ($n = 12$).

revealed a significant increase in neutralizing antibody titers by Day 14, which were further elevated following the boost immunization. By Day 21 (7 days post-boost), the vaccinated ducks developed substantial neutralizing antibody responses, with mean titers exceeding 1:80 (range: 1:64–1:128). These elevated titers persisted through Day 28, while control animals remained seronegative throughout the study period (titers $< 1:4$).

Intriguingly, we detected significant levels of neutralizing antibodies in the egg yolks harvested from immunized ducks, with mean titers of 1:24 (range: 1:16–1:32) (Fig. 4C). This represents approximately 25–30% of the corresponding maternal serum antibody levels, demonstrating efficient trans-ovarian transfer of protective antibodies. These findings suggest the potential for passive immunity transfer to offspring through egg yolk antibodies, a crucial consideration for vaccination strategies in commercial duck farming.

Discussion

This study revealed the efficacy superiority of a novel DTMUV mRNA vaccine over a commercial live-attenuated vaccine. Importantly, our mRNA vaccine has the potential to protect against highly virulent strains of DTMUV and exhibited efficient maternal antibody transfer through egg yolk deposition. Since 2020, the research and development of veterinary mRNA vaccines have been increasing¹⁷. However, most published researches were carried out on mammals, particularly swine, cats and dogs. Our study clearly demonstrated mRNA vaccines' feasibility on protecting poultry and proposed rational immunization schedules for poultry. Such inspiring findings will pave the way for safer and cleaner poultry farming.

The observed complete protection against the highly virulent JS804 strain represents a crucial breakthrough in DTMUV control, considering the complex immunological landscape of flavivirus infections. The robust neutralizing antibody responses was associated with protective efficacy, demonstrating mRNA vaccine's substantial advantages over live-attenuated vaccines. The superior efficacy can be attributed to the vaccine's capability of inducing potent humoral immune responses targeting critical epitopes for viral entry and fusion^{18–20}. A distinctive feature of mRNA vaccines lies in their enhanced cellular immune response. These vaccines facilitate antigen expression within host cells, where proteasomal degradation generates epitopes presented by MHC complexes, thereby inducing robust cellular immune responses. This mechanism further activates T follicular helper cells, which in turn stimulate B cell response, ultimately reinforcing humoral immune response—a significant advantage over inactivated vaccines^{21,22}. Moreover, mRNA vaccines circumvent the risks associated with genomic integration and avoid the adverse effects stemming from intracellular replication characteristic of attenuated vaccines. Further, unlike traditional live-attenuated vaccines that are often affected by interference from pre-existing immunity or incomplete attenuation⁷, our mRNA vaccine consistently induced high-titer neutralizing antibodies that strongly correlated with protection. This superior immunological profile, characterized by rapid antibody induction and complete protection against highly pathogenic strains, addresses the fundamental limitations of traditional DTMUV vaccination approaches.

The remarkable maternal antibody transfer observed in our study aligns with the fundamental mechanisms of avian immunoglobulin transport. The high levels of neutralizing antibodies detected in egg yolks suggest efficient transcytosis of IgY, the avian equivalent of mammalian IgG, across the follicular epithelium²³. Such process mediated by FcRY receptors ensures robust passive immunity in offspring, addressing a critical vulnerability window in early life when the immune system is still under development²⁴. The superior antibody titers achieved with our mRNA vaccine, compared to live attenuated alternatives, suggest enhanced humoral responses, potentially through optimal antigen presentation and robust expression of the immunogen.

In the context of emerging infectious diseases, our mRNA vaccine platform represents a crucial advancement in veterinary medicine's arsenal against DTMUV outbreaks. The devastating economic impact of DTMUV infections in the poultry industry, particularly in Asia-Pacific regions, underscores the urgent need for effective control strategies^{2,3}. DTMUV mRNA vaccine's capability of preventing both clinical disease and virus shedding suggests its potential to break transmission chains within flocks, offering a powerful tool for outbreak containment and disease eradication efforts. This comprehensive protection, combined with the inherent flexibility of mRNA vaccine technology to rapidly adapt to emerging virus strains, positions our vaccine as a revolutionary solution for controlling DTMUV outbreaks. The demonstration of complete protection against the prevalent JS804 strain particularly highlights its potential as a strategic intervention in regions experiencing active outbreaks or facing high risk of DTMUV emergence.

Looking forward, several critical aspects require further investigation to fully realize the potential of this vaccine platform. Long-term immunity studies will be essential to understand the durability of protection, particularly focusing on the retention of neutralizing antibodies and the potential role of memory B and T cell responses. The remarkable maternal antibody transfer observed opens new avenues for investigation into passive immunity dynamics in avian species. Future studies should address the duration of maternal antibody protection and its potential interference with active immunization in offspring. Additionally, while mRNA vaccines offer significant advantages in manufacturing scalability and strain adaptation, optimization of lipid nanoparticle formulations for enhanced thermostability will be crucial for practical field deployment.

Materials and methods

Study design

We conducted a series of controlled experiments to evaluate the efficacy and immunogenicity of our DTMUV mRNA vaccine. The study comprised three sequential phases: (1) immunization of SPF ducks ($n = 10$; 5 vaccinated, 5 controls) to assess protective efficacy against viral challenge, (2) comparative analysis in male breeder drakes ($n = 20$; 10 per group) to benchmark against a commercial live-attenuated vaccine (FX2010-180P), and (3) evaluation in laying ducks ($n = 12$; 6 vaccinated, 6 controls) to assess maternal antibody transfer. Blood samples were collected at indicated intervals for serological analyses, and egg yolks from vaccinated laying ducks were harvested for maternal antibody assessment.

mRNA vaccine design and construction

The mRNA vaccine encodes the pre-membrane (prM) and envelope (E) proteins of DTMUV. The both prM and E proteins are sourced from UniprotKB (Accession No. G1CRV4). The N-terminus of prM and E proteins is fused with a signal peptide from *Aythya fuligula* (Tufted duck) CD5 protein (UniprotKB Accession No. A0A6J3D041) to enhance the efficiency of protein expression and secretion. This design, inspired by the successful strategies of Zika and dengue mRNA vaccine^{15,16}, endeavors to recapitulate the native viral structure. The mRNA construct incorporates optimized 5' and 3' untranslated regions, poly(A) tail of approximately 110 nucleotides to enhance mRNA stability and translation efficiency, a type 1 cap structure, and the duck CD5 signal peptide to enhance expression and secretion efficiency. For plasmid construction, the pUC57 vector (sourced from GenScript) was utilized to facilitate *in vitro* mRNA transcription. The T7 promoter element was additionally introduced into the pUC57 vector to enable efficient *in vitro* mRNA transcription.

mRNA *in vitro* transcription and purification

mRNA was transcribed from linearized plasmid DNA using the MEGAscript® T7 Transcription Kit, with CleanCap (Cat# N-7413, TriLink), a trinucleotide cap1 analog for co-transcription. After a 6-h *in vitro* transcription reaction, mRNA was selectively precipitated using lithium chloride. The resulting mRNA pellet was dissolved in nuclease- and endotoxin-free water, followed by quantification and quality control using a Nanodrop spectrophotometer and capillary electrophoresis, respectively. Finally, the purified mRNA was aliquoted and stored at -80°C .

Western blot analysis

293 T cells, seeded at a density of 3.75×10^6 cells per T75 flask, underwent transient transfection with naked mRNA using a lipid nanoformulation named LNP3²⁵, which was previously optimized for effective mRNA delivery. At 48 h post-transfection, the cell supernatant was collected and concentrated using an ultrafiltration tube, while the cells were harvested for lysis and protein extraction using the RIPA method. The lysis buffer contained 20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 $\mu\text{g}/\text{mL}$ aprotinin, and 1 mM phenylmethanesulfonyl fluoride. The protein concentrations of both the concentrated supernatant and the cell lysate were determined using the BCA method. Subsequently, 40 μg of total protein from each sample was subjected to SDS-PAGE separation and then transferred onto PVDF membranes for further analysis. The membranes were blocked overnight at 4°C with 5% skim milk in a phosphate buffer (pH 7.2) containing 0.05% Tween-20. Then membranes were probed with a primary anti-DTMUV-E polyclonal antibody (mouse origin), which was diluted 1:200 in the same blocking solution. This antibody was generously provided by Dr. Qingtao Liu's laboratory (Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biological Engineering and Technology, Ministry of Agriculture; Jiangsu Key Laboratory for Food Quality and Safety-State Key Laboratory Cultivation Base of Ministry of Science and Technology, Nanjing, China) and used at room temperature for one hour. After washing three times with PBST, the membranes were exposed to a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody, diluted 1:2000 in 5% milk-PBST, for an hour at room temperature. Post three additional washes, the protein bands were visualized using the ChemiDocTM system (Bio-Rad, USA) and ImmunoStar™ HRP substrate (Bio-Rad, USA) for chemiluminescence detection.

mRNA-LNP production

Lipid nanoparticles encapsulating mRNA (mRNA-LNPs) were produced using microfluidic technology. Lipids were dissolved at a molar ratio of 50:10:38.5:1.5 (SM102: DSPC: cholesterol: DMG-PEG2000). mRNA was prepared at a concentration of 0.1 mg/mL in 20 mM sodium acetate buffer (pH 5.5). The lipid and mRNA solutions were then mixed using a microfluidic cartridge at a total flow rate of 6 mL/min, with an aqueous to organic phase flow rate ratio of 3:1, resulting in an N/P ratio of 6. The obtained mRNA-LNPs were dialyzed three times against 20 mM Tris-HCl buffer

(pH 7.4) using a Slide-A-Lyzer dialysis cassette with a 3.5 kD molecular weight cut-off. The final mRNA-LNP solution was adjusted to a concentration of 100 $\mu\text{g}/\text{mL}$ in 10% sucrose (w/v) and aliquoted for storage under $2-8^{\circ}\text{C}$ conditions.

Quality control of mRNA-LNP complex

To ensure the integrity and performance of the mRNA-LNP complexes, comprehensive quality control measures were implemented. The size and polydispersity index (PDI) of the mRNA-LNPs were measured using dynamic light scattering (DLS). The particles exhibited a mean diameter ranging from 60 to 200 nm, with a polydispersity index (PDI) consistently below 0.2, indicating a narrow size distribution and high uniformity. The encapsulation efficiency of the mRNA was determined using the RiboGreen assay, and the results demonstrated that the mRNA-LNPs achieved an encapsulation efficiency greater than 80%, ensuring effective protection of the mRNA within the lipid shell.

Ethics and bio-containment statement

The conducted animal experiments received authorization from the Institutional Animal Care and Use Committee at Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences. All procedures involving animals were in strict compliance with ARRIVE reporting guidelines²⁶. Experiments involving the DTMUV were conducted at Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences within a bio-containment unit located in a BSL-2 laboratory to ensure safety and containment.

Vaccination and challenge experiments

All experimental procedures were conducted using Jinding ducks, a domesticated breed obtained from the Harbin Veterinary Research Institute in China. At the conclusion of each experiment, ducks were humanely euthanized by intravenous injection of sodium pentobarbital at a dose of 100 mg/kg of body weight in accordance with institutional and national guidelines for the ethical treatment of experimental animals.

For the immunization of SPF ducks, initial priming was conducted when ducks were 7 days old, followed by a booster dose at 28 days. Each vaccination involved a 5 μg dose of the formulated mRNA vaccine delivered via intramuscular injection. Sampling for immunological assays was scheduled at days 28, 35, 42, and 49. For the challenge experiments, ducks received exposure to the highly pathogenic DTMUV strain JS804 (GeneBank: JF895923) at day 49 post-prime vaccination, with an infection dosage of 10^5 TCID₅₀ in a volume of 0.5 ml. Protection after challenge was then monitored.

To compare the efficacy of our mRNA vaccine with commercial live attenuated vaccine (strain FX2010-180P), male breeder drakes were vaccinated at day 0 (prime) and subsequently received a booster dose on day 21. Each dose consisted of 10 μg of the vaccine administered via intramuscular injection. Blood samples were collected on days 21 and 28 post-initial vaccination to measure the levels of specific and neutralizing antibodies.

For the immunization of laying ducks, initial priming was conducted at day 0, followed by a booster dose at day 14. Each vaccination involved a 5 μg dose of the formulated mRNA vaccine delivered via intramuscular injection. Blood samples were collected on days 0, 14, 21, and 28 to measure the levels of neutralizing antibodies. Neutralizing antibodies in egg yolks were also determined. The laying ducks used were Jinding Ducks (JDD), selected for their high egg production and suitability for vaccine efficacy studies. They were maintained under controlled breeding conditions with a balanced diet to ensure optimal health and consistent egg quality.

Determination of anti-DTMUV E protein IgY antibodies

Serum specific E protein IgY were detected using commercial ELISA kits (Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences) following the manufacturers' protocol. According to the manufacturer's instructions, the ELISA titer is the highest dilution factor at which serum sample is determined to be positive. Through linear regression analysis, there is a certain correlation between the logarithm of the reciprocal of the serum ELISA titer and the OD value of the serum at a 1:10 dilution.

A standard curve is plotted using the OD value of serum at a 1:10 dilution as x-axis and $\log [1/ET]$ as y-axis ($\log [1/ET] = a + bx$, a and b respectively represent the intercept and slope of the linear regression equation, and x represents the OD value of the serum to be tested). The titer of each serum can be calculated from the regression equation.

Neutralization assay

Serum samples were collected, inactivated in a 56 °C water bath for 30 min, and diluted two-fold in Minimum Essential Media (Thermo Fisher). Equal volumes of serum dilutions were mixed with 100-fold diluted 50% tissue culture infective dose (TCID₅₀) of DTMUV and incubated in 96-well plates at 37 °C for one hour. BHK-21 cells were then seeded into each well and co-cultured with the mixture for five days. Neutralizing antibody titers were assessed by monitoring the cytopathic effect (CPE): neutralizing activity was confirmed when at least two out of three wells showed no CPE.

Statistical analysis

Data were analyzed using appropriate statistical methods where indicated. Differences between groups were considered significant at $*P < 0.05$. Graphical representations of antibody titers and protection rates were generated to visualize the results.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. The mRNA vaccine sequences used in this study have been submitted to GenBank with the ID: PV470651. The sequence is publicly accessible via the following URL: <https://www.ncbi.nlm.nih.gov/nucleotide/PV470651.1/>.

Received: 24 October 2024; Accepted: 28 April 2025;

Published online: 05 May 2025

References

- Hamel, R. et al. New insights into the biology of the emerging Tembusu virus. *Pathogens* **10**, 1010 (2021).
- Zhang, W., Chen, S., Mahalingam, S., Wang, M. & Cheng, A. An updated review of avian-origin Tembusu virus: a newly emerging avian Flavivirus. *J. Gen. Virol.* **98**, 2413–2420 (2017).
- Lei, W. et al. The genetic characteristics and evolution of Tembusu virus. *Vet. Microbiol.* **201**, 32–41 (2017).
- Cheng, Y. et al. Advancements in research on Duck Tembusu Virus infections. *Viruses* **16**, 811 (2024).
- Zhang, L. et al. Efficacy assessment of an inactivated Tembusu virus vaccine candidate in ducks. *Res. Vet. Sci.* **110**, 72–78 (2017).
- Lv, J. et al. Detection of neutralizing antibodies to Tembusu Virus: implications for infection and immunity. *Front. Vet. Sci.* **6**, 442 (2019).
- He, D., Zhang, X., Chen, L., Tang, Y. & Diao, Y. Development of an attenuated live vaccine candidate of duck Tembusu virus strain. *Vet. Microbiol.* **231**, 218–225 (2019).
- Tang, J. et al. Immunization with a suicidal DNA vaccine expressing the E glycoprotein protects ducklings against duck Tembusu virus. *Virol. J.* **15**, 140 (2018).
- Nielsen, H. S. et al. Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. *J. Gen. Virol.* **82**, 1263–1272 (2001).
- Zhou, B. et al. Reversion of cold-adapted live attenuated influenza vaccine into a pathogenic virus. *J. Virol.* **90**, 8454–8463 (2016).
- Ma, T. et al. Liposomes containing recombinant E protein vaccine against duck Tembusu virus in ducks. *Vaccine* **34**, 2157–2163 (2016).
- Yu, K. et al. Structural, antigenic, and evolutionary characterizations of the envelope protein of newly emerging Duck Tembusu Virus. *PLoS ONE* **8**, e71319 (2013).
- Pardi, N., Hogan, M. J., Porter, F. W. & Weissman, D. mRNA vaccines – a new era in vaccinology. *Nat. Rev. Drug Discov.* **17**, 261–279 (2018).

- Hogan, M. J. & Pardi, N. mRNA vaccines in the COVID-19 pandemic and beyond. *Annu. Rev. Med.* **73**, 17–39 (2022).
- Richner, J. M. et al. Modified mRNA vaccines protect against Zika Virus Infection. *Cell* **168**, 1114–25.e10 (2017).
- Wollner, C. J. et al. A dengue virus Serotype 1 mRNA-LNP vaccine elicits protective immune responses. *J. Virol.* **95**, 10–1128 (2021).
- Fazel, F. et al. The mRNA vaccine platform for veterinary species. *Vet. Immunol. Immunopathol.* **274**, 110803 (2024).
- Shaozhou, W. et al. Duck tembusu virus and its envelope protein induce programmed cell death. *Virus genes* **51**, 39–44 (2015).
- Chen, X. et al. A novel neutralizing antibody targeting a unique cross-reactive epitope on the hi Loop of Domain II of the envelope protein protects mice against Duck Tembusu Virus. *J. Immunol.* **204**, 1836–1848 (2020).
- Huang, J. et al. Oral delivery of a DNA vaccine expressing the PrM and E genes: a promising vaccine strategy against flavivirus in Ducks. *Sci. Rep.* **8**, 12360 (2018).
- Lee, J., Woodruff, M. C., Kim, E. H. & Nam, J. H. Knife's edge: balancing immunogenicity and reactogenicity in mRNA vaccines. *Exp. Mol. Med.* **55**, 1305–1313 (2023).
- Lederer, K. et al. SARS-CoV-2 mRNA vaccines foster potent antigen-specific germinal center responses associated with neutralizing antibody generation. *Immunity* **53**, 1281–1295.e5 (2020).
- Counihan, K. L., Maniscalco, J. M., Bozza, M., Hendon, J. M. & Hollmen, T. E. The influence of year, laying date, egg fertility and incubation, individual hen, hen age and mass and clutch size on maternal immunoglobulin Y concentration in captive Steller's and spectacled eider egg yolk. *Dev. Comp. Immunol.* **52**, 10–16 (2015).
- Okamoto, M. et al. FcRY is a key molecule controlling maternal blood IgY transfer to yolks during egg development in avian species. *Front. Immunol.* **15**, 1305587 (2024).
- Chen, H. L., Ren, X., Xu, S., Zhang, D. K. & Han, T. Y. Optimization of lipid nanoformulations for effective mRNA delivery. *Int. J. Nanomed.* **17**, 2893–2905 (2022).
- Percie du Sert, N., Hurst, V. & Ahluwalia, A. et al. The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *BMC. Vet. Res.* **16**, 242 (2020).

Acknowledgements

This work was supported by Jiangsu Provincial Agricultural Science and Technology Innovation Foundation[No. CX(21)3134].

Author contributions

Q.L. and T.H. conceived and supervised the project. S.X. and L.Z. designed and performed the majority of experiments, analyzed data, and drafted the manuscript. C.F. and G.L. assisted with the mRNA vaccine construction and LNP formulation. M.X. and R.L. conducted the animal immunization experiments and challenge studies. J.L. and A.W. performed the neutralizing antibody assays. Q.H. and K.C. contributed to data analysis and interpretation. Q.L. and T.H. reviewed and edited the manuscript. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to Qingtao Liu or Tiyun Han.

Reprints and permissions information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License, which permits any non-commercial use, sharing, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if you modified the licensed material. You do not have permission under this licence to share adapted material derived from this article or parts of it. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>.

© The Author(s) 2025