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Development of chimeric virus-like particles containing the E glycoprotein of duck Tembusu virus

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ABSTRACT

Duck Tembusu virus (DTMUV) has caused enormous economic losses to the poultry industry in China. In the current study, we generated chimeric virus-like particles (VLPs) containing E protein of the DTMUV and HA2 protein of the H3N2 avian influenza virus (AIV). The chimeric VLPs could induce specific antibody responses in both mice (n = 5/group) and ducks (n = 10/group). After immunizing ducklings with the chimeric VLPs, all immunized ducks (n = 10/group) were 100% (10/10) protected against homologous DTMUV strain and virus shedding was not detected on day 5 post-challenge, whereas 60% (6/10) of the ducklings immunized with PBS presented typical symptoms with a virus shedding rate of 90% (9/10). Furthermore, viral loads were significantly decreased in the birds of the chimeric VLPs immunized group, comparing to that of the PBS immunized group. Our data demonstrated that the chimeric VLPs used in the current study could be applied as a potential vaccine candidate to control DTMUV infections in young ducks.

1. Introduction

Since the first report of duck Tembusu virus (DTMUV) in China in April 2010, the virus has caused significant economic losses to poultry industry (Cao et al., 2011; Li et al., 2013; Yan et al., 2011). The infected duck experience rapid egg drop ranging from 20% to 90%, delayed growth, and neurological symptoms (Zhang et al., 2017). The morbidity rate is approximately 100%, whereas the mortality rate varies from 5% to 30% (Yan et al., 2011). DTMUV belongs to the genus *Flavivirus*, family Flaviviridae. As other flaviviruses, DTMUV is a mosquito-borne virus (Tang et al., 2015) and can infect many species. In addition to fowl species such as duck, goose (Ti et al., 2015), chicken (Chen et al., 2014b), and pigeon (Dai et al., 2015), species such as sparrow (Tang et al., 2013) can also be infected by the virus. Besides China, outbreaks of DTMUV infections have also been reported in Malaysia and Thailand during the monsoon season (July–December) since 2012 (Thontiravong et al., 2015). To date, though commercial vaccines have been developed, this contagious disease has led to huge economic losses to the poultry industry in the Southeast Asia (Benzarti et al., 2019).

The viral genome consists of a single-stranded, positive-sense RNA of approximately 11 kb in length with a long open reading frame (ORF) encoding a large polyprotein (Liu et al., 2013a,b). The polyprotein is further cleaved into several structural proteins: capsid (C), membrane (M), and envelope (E) proteins, and seven nonstructural proteins (Liu et al., 2013a,b). The envelope (E) protein, which is considered to be the primary immunogen of the flaviviruses, is a glycoprotein locating on virion surface (Heinz and Stiasny, 2012). Similar to other flaviviruses (such as Japanese encephalitis virus), the ectodomain of the E protein contains three distinct domains, including envelope domain I (EDI), EDII, and EDIII. Moreover, the protein possesses numerous T cell and B cell epitopes which can elicit both humoral and cell-mediated immune response. Furthermore, E protein plays important roles in virus receptor binding, host specificity, cell tropism, and virulence (Li et al., 2016). Therefore, based on the E protein, many flavivirus vaccines have been developed (Heinz and Stiasny, 2012; Ma et al., 2016). It was reported that recombinant Goose TMUV E protein could induce significant humoral and cell-mediated responses in female SPF ducks (Zhao et al., 2015a), and liposome vaccines containing recombinant DTMUV E

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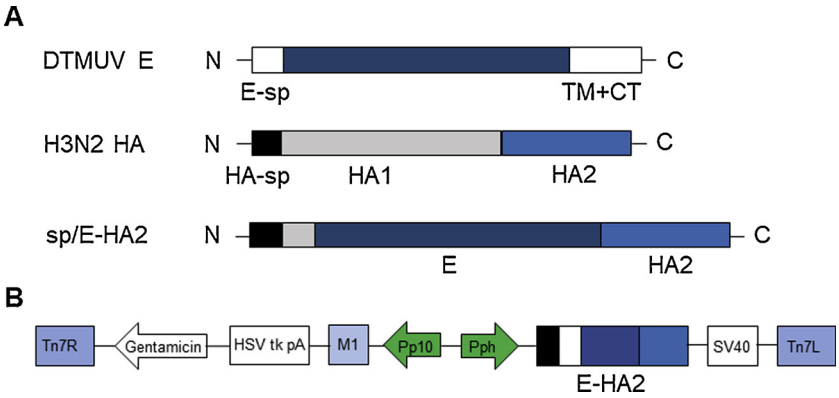

Fig. 1. Simplified schematic diagram of chimeric VLPs E-HA. (A) Structural schematic diagram of E, HA and fused E-HA2 proteins. (B) Schematic diagram of rBVs. The pFastBacDual vector was used. The E-HA2 was under the control of polyhedron (PH) promoter and the M1 was under the control of p10 promoter.

protein could provide complete protection to prevent DTMUV infection in cherry valley ducks (Ma et al., 2016).

Virus-like particles (VLPs) have been proven to be a safe and effective vaccine candidate and have been used for prevention of infections of influenza virus (IV), hepatitis E virus (HEV), etc (Haynes, 2009; Roldao et al., 2010). Flavivirus VLPs containing structural proteins such as the E proteins, have been produced in preclinical or early stage clinical studies, take West Nile virus, Japanese encephalitis virus, Tick-borne encephalitis virus, and Zika virus and Dengue virus for instance (Boigard et al., 2017; Ferlenghi et al., 2001; Pijlman, 2015; Urakami et al., 2017; Yamaji and Konishi, 2016). In influenza virus, the HA protein serves as the predominant antigenic protein of the influenza virus, the highly conserved subunit HA2 of which contributes to trimerization and HA stability (Kemble et al., 1993; Wang et al., 2015). The matrix proteins in enveloped viruses, such as the M1 protein in the influenza virus, plays a crucial role in virus budding and virus particle stabilization (Liljeroos et al., 2013). So far, influenza VLPs platform were well developed, and VLPs (containing the HA2 and M1 proteins) have been shown to be efficient inducers of immune responses in mice (Qin et al., 2018). Previous research demonstrated that the reconstructed HA containing a transmembrane (TM) domain of the H3N2 subtype showed an increased thermal stability and immunogenicity (Liu et al., 2015; Qin et al., 2018).

So far, VLPs based on DTMUV structural proteins have not been reported. To take the advantages of genetic engineering vaccines, VLPs containing the E protein would serve as a promising vaccine candidate. Furthermore, since DTMUV E protein also contains a transmembrane domain, it is intriguing for us to know whether VLPs containing recombinant H3N2 hemagglutinin and DTMUV E protein could serve as a platform for the DTMUV E protein-based vaccine design, and whether this VLPs induce better or similar immunogenicity and protective efficacy against DTMUV infections. In the current research, we constructed a fused protein in which the DTMUV E protein was fused with the signal peptides of influenza H3N2 HA in the 5′end and the HA2 domain of influenza H3N2 HA protein in the 3′end (HA2 domain contains the transmembrane domain(TM) and cytoplasmic tail (CT)of HA protein). The results showed that the co-expression with fusion protein E-HA2 and H3N2 M1 protein could efficiently assemble to form chimeric VLPs. These chimeric VLPs were then used as immunogens in BALB/c mice and female SPF ducks. When compared with the DTMUV inactivated strain, the chimeric VLPs induced similar immune responses and provided good protection as the inactivated strain.

2. Materials and methods

2.1. Ethics statements

This work was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of People's Republic of China. The animal study was supervised by the Committee on the Ethics of Animal Experiments of Institute of Animal Health, Guangdong Academy of Agricultural Sciences Experimental Animal Welfare Ethics Committee (Approve ID: 2018-009.) and used in accordance with the regulation and guidelines of this committee.

2.2. Cells and viruses

Sf9 insect cells were cultured in serum-free SF900II medium (GIBCO, Grand Island, NY, USA) at 27 ± 0.5℃ in spinner flasks with a speed of 100 × *g*. Duck Tembusu virus strain JM (GenBank accession numbers JN811559) was isolated and stored in our laboratory, and propagated in nine-day-old specific pathogen free (SPF) duck embryos (was purchased from Harbin Veterinary Research Institute, Harbin, China). The influenza H3N2 strain (A/swine/Guangdong/01/1998) was kindly provided by Prof. Yongchang Cao (Sun Yat-sen University, Guangzhou, China).

2.3. Construction of recombinant baculoviruses rBV- E-HA2-M1

DTMUV E gene from JM strain lacking the signal peptide and transmembrane domain (1047bp), signal peptide (sp) of HA gene, HA2 from H3N2 strain (A/swine/Guangdong/01/1998) (GenBank accession number FJ830855.1) were first amplified and cloned into pMD-18 T vector (Takara, Dalian, China) and then sequenced (Invitrogen, Guangzhou, China). Plasmid pFastBac™Dual-M1, in which H3N2 M1 were cloned into the pFastBac™Dual baculovirus transfer vector (Qin et al., 2018), was gifted by Prof. Yongchang Cao (Sun Yat-sen University, Guangzhou, China). The sp-E-HA2 fusion genes were then generated by overlapped PCR and cloned between the Spe I and Hind III sites of pFastBac™Dual-M1 vector to generate recombinant shuttle plasmids (Fig. 1A). The shuttle vectors were then chemically transformed into competent DH10Bac™ *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA). All clones were verified by sequencing (Invitrogen, Guangzhou, China). Generation of recombinant baculovirus was performed as previously described (Liu et al., 2015). Briefly, the obtained purified recombinant bacmids were transfected into Sf9 cells with cellfectin reagent and incubated for 3 days. The target recombinant baculoviruses (rBV) were then harvested from the supernatant. The structure of the recombinant fusion proteins is designated as rBV-E-HA2 (Fig. 1B). All primers used in this study were synthesized by Invitrogen and summarized in Table1.

2.4. Western-blot

Sf9 cells at a density of 1×10^6 cells per flask were infected with

Table 1
Details of PCR primers used in cloning.

Primer pairs	Sequence (5′–3′)	Size (bp)
H3N2 HA-sp	ATGAAGACTATCATTGCTTTG CTGAACCAGTTCAGTAGCAT	180
DTMUV E	GAATTAGCGGTTGTGAGATCT ACTTCTATGCCACTGGTACCT	1047
H3N2 HA-HA2	GGCATATTCGGCGCAA TCAGATGCAAATGTTGCACC	666
E-HA2	GGACTAGTATGAAGACTATCATTGCTTT CCAAGCTTTCAGATGCAAATGTTGCACC	1893

rBV-E-HA2 at a multiplicity of infection (MOI) of 5. After 72 h, obvious cytopathic effects were observed. The infected cells were then harvested and cell lysates were separated on 10% SDS polyacrylamide gels, and then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were blocked and subsequently detected with mouse polyclonal sera against DTMUV virus (Sun et al., 2018) at a 1:2000 dilution, chicken polyclonal sera against H3N2 virus (Wen' s Foodstuffs Group Co., Ltd, China) at a 1:2000 dilution, and mouse anti-H3 HA monoclonal antibody (Abcam, Cambridge, UK) at a 1:2000 dilution, respectively. Horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody and HRP-conjugated anti-chicken secondary antibody was used at a dilution of 1:8000 and 1:5000 (Proteintech Group, Rosemont, IL, USA), respectively.

2.5. Indirect immunofluorescence

Sf9 cells at a density of 1×10^6 cells/ml were cultivated on 12-microwell plates (Greiner Bio-One, Germany) at 28 ℃, and infected with the rBV-E-HA2 at a MOI of 1 for 48 h. Cells were washed three times with PBS-Tween-20 (0.05%) and fixed with 4% formaldehyde at room temperature. Mouse polyclonal sera against DTMUV virus (1:2000), mouse anti-H3 HA monoclonal antibody (1:2000), and rabbit anti-M1 polyclonal antibody (kindly provided by Prof. Yongchang Cao, 1:2000.) were applied as primary antibody, respectively, and incubated with cells for 1 h at room temperature. The cells were then subsequentially stained with FITC-conjugated rabbit anti-mouse secondary antibody (Beijing Biosynthesis Biotechnology, Beijing, China) and Cy3-conjugated goat anti-rabbit secondary antibody (Proteintech Group, Rosemont, IL, USA). The cell nuclei were stained with DAPI (Beijing Biosynthesis Biotechnology, China). TCS-SP5 confocal laser scanning microscope (Leica, Germany) was applied for further analysis.

2.6. Preparation of chimeric VLPs

To produce chimeric VLPs containing the fusion proteins, Sf9 cells were infected with rBV-E-HA2 at a MOI of 5. After incubation with serum-free SF900II medium (GIBCO, Grand Island, NY, USA) for 72 h at 28 ℃ at a speed of 100 g, culture supernatants were harvested and the VLPs were purified using 20%–30%–60% (w/v) discontinuous sucrose gradient ultracentrifugation at 100,000 × *g* for 2 h at 4 ℃. The fractions were collected and the presence of fusion protein E-HA2 and M1 protein was analyzed by western blot.

2.7. Electron microscopy

The purified VLPs were analyzed by electron microcopy. Briefly, 200-mesh carbon-coated copper grids were floated on drops of chimeric VLPs for 10 min, then the grid was washed and stained with 2% phosphotungstic acid (pH 7.0) for 1 min. After the girds were air-dried, the stained VLPs were observed by transmission electron microscopy (JEM-100CX-II; JEOL, Japan).

2.8. Animals, immunization, and viral challenge

Fifteen six-week-old female BALB/c mice divided into three groups (n = 5/group) were purchased from Laboratory Animal Center of Southern Medical University and immunized intramuscularly (i.m.) with chimeric VLPs, inactivated DTMUV JM strain or PBS two times (at week 0 and 2, respectively) at a two-week interval. chimeric VLPs (4 μg of E protein/dose) were applied for the primary immunization (at weeks 0) with complete Freund's adjuvants (CFA), or incomplete Freund's adjuvant (IFA) for subsequent booster immunization (weeks 2). The DTMUV JM strain (containing 4 μg of E protein/dosage) was inactivated using 0.2% formaldehyde solution (V/V) and emulsified with white oil. Serum samples were collected at different time points (before immunization, on the 7th, 14th day after primary immunization, on the 7th, 14th, 21 st day after booster immunization).

Thirty ten-day-old SPF ducks were purchased (Harbin Veterinary Research Institute, Harbin, China) and housed in individual isolators under positive pressure. They were randomly divided into three groups (n = 10/group). SPF ducks were immunized intramuscularly (i.m.) with chimeric VLPs (Each dose contained 4 μg of E protein emulsified with Montanide™ ISA 71 V G as recommended by the manufacturer (Seppic, Paris, France)), inactivated DTMUV JM strain (containing 4 μg of E protein/dosage) or PBS two times (at week 0 and 2 post immunization) as above described. Serum samples were collected at different time points (before immunization, on the 7th, 14th day after primary immunization, on the 7th,14th, 21 st day after booster immunization) and stored at −80℃. Three weeks after immunization, vaccinated ducks were challenged intramuscularly with $10^{3.0}EID_{50}$ of DTMUV JM strain (GenBank accession no. JN811559), as the dosage of $10^{3.0}EID_{50}$ of the strain caused high morbidity in young ducks (preliminary data). After challenge, the ducks were observed for clinical signs including anorexia, diarrhea, and reluctance to move for 14 days, cloacal swabs were collected from ducks on day 5 post-challenge for RNA extraction and all ducks were weighed on day 14 post-challenge.

2.9. ELISA

The DTMUV virus-specific antibodies were determined in the mice sera samples and duck sera samples by an indirect Enzyme-Linked Immunosorbent Assay (ELISA). E protein were purified from *E. coli* Rosseta transforming with pET32a-E, and used as antigen to detect the DTMUV-specific antibodies. Purified E protein with a concentration of 3 μg/ml were coated, and incubated with serum samples from immunized mice or from immunized SPF ducks at a 100-fold dilution for 1 h. HRP-conjugated rabbit anti-mouse secondary antibodies (Proteintech Group, Rosemont, IL, USA) diluted 8000-fold were added and incubated for 1 h for murine sera, and HRP-conjugated donkey anti-duck secondary antibodies (Proteintech Group, Rosemont, IL, USA) diluted 5000-fold were added and incubated for 1 h for duck sera, respectively. Signals were visualized with ELISA substrate reagents and measured at 450 nm.

2.10. Real time RT-PCR

To examine viral replication ability in E-HA2 vaccinated group and the control group, 3 ducklings in each group were sacrificed at different time points (2, 4, 6, 8, and 10 dpc). Spleens, livers, kidneys, and brains were collected to detect viral loads.

cDNA was obtained by reverse transcription using a PrimerScript® RT Master Mix Perfect Real Time kit (TaKaRa, Shiga, Japan) according to the manufacturer's instruction. Real time RT-PCR was perform as described before (Liu et al., 2013a,b). All experiments were performed in triplicate.

2.11. Histopathology

The duck spleen, liver, and kidney samples were collected at day 6 post challenge, fixed in 10% formalin and embedded in paraffin wax. Then, they were cut into 5 μm thin sections and stained with hematoxylin and eosin. The slides were examined with light microscopy (Leica, Germany) for lesions.

2.12. Statistical analysis

Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). Two-tailed Student's t-tests were applied for statistical analysis. Statistical difference between two groups was indicated by **** ($P < 0.00001$).

3. Results

3.1. Expression of fusion proteins E-HA2 and M1 protein in Sf9 cells

To construct the fused E-HA2 protein, recombinant E protein was fused to the signal peptides, 180 bp of influenza H3N2 HA gene in the N termina,l and the HA2 domain of H3N2 HA protein in the C terminal (Fig. 1A). Recombinant baculoviruses (rBVs) expressing chimeric E-HA2 and H3N2 M1 proteins were generated using the pFastBac™ Dual bacmid transfer vector containing the AcMNPV polyhedrin promoter and p10 promoter (Fig. 1B).

To check whether the recombinant E-HA2 and M1 could be expressed in the Sf9 cells, we analyzed protein expression in collected cell lysates from the recombinant baculovirus-infected Sf9 cells. Western blot under denaturing condition showed that E-HA2 and M1 proteins were detectable with molecular weight of 80 kDa (Fig. 2A, B) and 28 kDa (Fig. 2A), respectively. These constructs were used later for VLPs preparation in our study.

3.2. Production and analysis of chimeric VLPs

Chimeric VLPs were automatically assembled and secreted from infected cells into the culture medium. Negatively stained VLPs-E-HA2 presented round shape with a diameter ranging from 80 to 100 nm, showing spikes on the viral particle surfaces. The inside of the VLPs was empty (Fig. 2C). Applying indirect immuno-fluorescence assays, the fused E-HA2 protein was localized at the surface of the infected sf9 cells, and the M1 protein was found in both cytoplasm and nucleus (Fig. 2D, E). The chimeric VLPs were purified by discontinuous sucrose density gradients.

3.3. Antibody responses of chimeric VLPs in mice

The immunogenicity of the chimeric VLPs (in presence of Freund's adjuvants) was evaluated in a mouse immunization trial, and inactivated DTMUV JM strain was used as a positive control. Mouse serum samples were collected before immunization, on the 7th, 14th day after primary immunization, and 7th,14th,21 st day after booster immunization (Fig. 3A). Serum was used to analyze E-specific IgG antibodies by ELISA. DTMUV E protein, as the major surface antigen of chimeric VLPs and DTMUV vaccine, was selected for the antigen of ELISA experiments. Birds in both chimeric VLP and inactivated vaccine groups showed significantly higher E-specific antibody responses than the PBS group ($p < 0.00001$; Fig. 3B). The antibody level of the chimeric VLPs and inactivated vaccine groups was similar ($p > 0.05$; Fig. 3B).

3.4. Chimeric VLPs provided protection in SPF ducks

Serum samples were collected before immunization, on the 7th, 14th day after primary immunization, and on the 7th, 14th, 21 st day after booster immunization for analysis of E-specific antibody responses of DTMUV (Fig. 4A). Pathological sections of the spleens of all three groups were examined and no pathological changes were observed in the immunized group (Fig. 4B). DTMUV E protein was utilized as ELISA antigen. Similar as the antibody response results in mice, we found that the group of SPF ducks inoculated with chimeric VLPs and inactivated DTMUV strain displayed no significant differences in the E-specific antibody responses (Fig. 4C). Both chimeric VLPs and inactivated DTMUV strain immunized groups displayed significantly enhanced levels of the E-specific antibody responses in contrast to that of the PBS group ($p < 0.00001$, Fig. 4C). These results suggest that the immunization with chimeric VLPs could induce specific antibodies to DTMUV in SPF ducks.

To determine the level of protection provided by the chimeric VLPs, SPF ducks were challenged with $10^{3.0}$ EID_{50} of homologous highly virulent DTMUV strain three weeks after the boost immunization and observed daily for 14 days to monitor clinical symptoms. After challenge, comparing to the immunized group using inactivated DTMUV vaccine, ducks in PBS group showed obvious weight loss (Table 2), whereas the chimeric VLPs groups presented no significance. Furthermore, typical symptoms within four days after challenge were observed only in PBS group, where 60% of the birds showed reduced intake (6/10), 50% showed diarrhea (5/10) and 60% showed reluctance to move (6/10). Virus shedding was detected in 90% of the birds (9/10) on day 5 post-challenge (Table 3), while slight illness symptoms were observed in ducks immunized with the chimeric VLPs and inactivated DTMUV strain. Ducks immunized with the inactivated DTMUV strain presented only mild clinical signs of anorexia in the first few days and recovered quickly (Table 3). Ducks immunized with the chimeric VLPs presented similar mild symptoms as theinactivated group. 10% of the ducks (1/10) in both VLPs and inactivated vaccine groups presented deceasing in intake, as well as diarrhea, while 10% of the ducks in inactivated strain group (1/10) presented reluctance to move in the first 4 days. No virus shedding was detected in both immunized groups on day 5 post-challenge (Table 3). Furthermore, to further evaluate the efficacy of the chimeric VLPs, viral loads of different organs (kidney, brain, liver, and spleen) were analyzed with quantitative RT-PCR. The viral loads of different organs of the chimeric VLPs immunized ducks were significantly decreased from the second day after challenge (Fig. 5A). Pathological section of the livers and kidneys were examined. The samples of PBS inoculated group presented pathological lesions, such as hemorrhage and tubular dilation, while samples of the chimeric VLPs inoculated group did not present any pathological damages (Fig. 5B).

Taken together, these data suggest that the chimeric VLPs could promote immune protection against DTMUV JM challenge.

4. Discussion

As a newly emerged avian flavivirus, DTMUV causes hemorrhaging, degeneration and lymphocyte infiltration of the ovaries, thus resulting in symptoms such as sudden egg-dropping in layer ducks, as well as high mortality and retarded growth in meat ducks. Though inactivated and attenuated live DTMUV vaccines have been applied in China, the outbreaks of the highly contagious disease among domestic fowl have resulted in greater economic losses. As the major antigen of the virus, the DTMUV E protein contributes as the target protein in vaccine development. For instance, recombinant viruses (NDV (Sun et al., 2018), ADV (Tang et al., 2019), DEV (Chen et al., 2014a), JEV (Wang et al., 2016) containing DTMUV E protein can completely protected ducks from DTMUV challenge.

Due to the problem of antibody dependent enhancement with inactivated vaccines and the biosecurity problems caused by attenuated live vaccines (Zhao et al., 2015b), it is urging to develop a more effective and affordable vaccine with more security against DTMUV. As other flavivirus, the design strategy of the genetically engineered DTMUV vaccine is focused on the E glycoprotein (Heinz and Stiasny, 2012; Ma et al., 2016). For instance, *E coli* expressed recombinant E

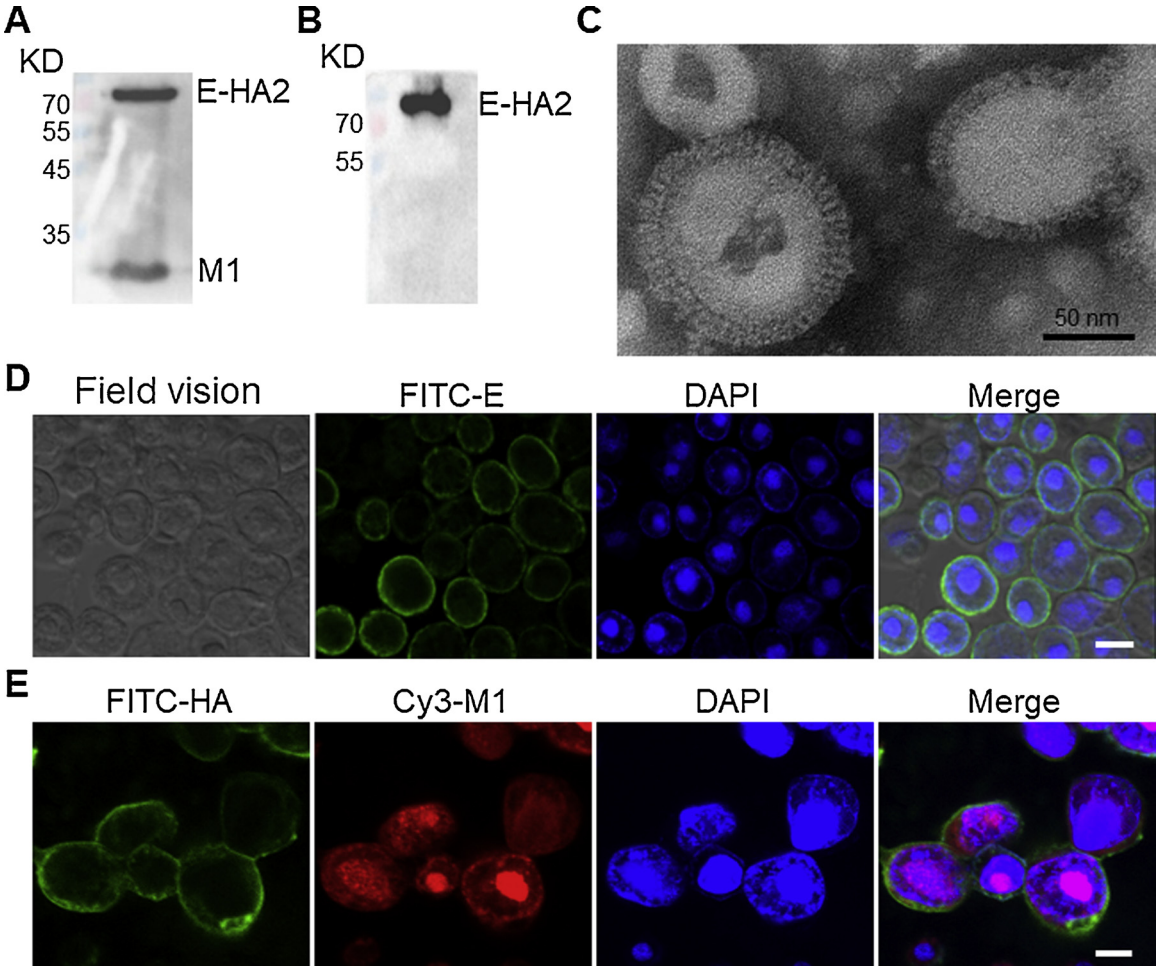

Fig. 2. Identification of the chimeric VLPs E-HA2. (A) Western Blot analysis of HA and M1 proteins in Sf9 cells applying chicken polyclonal sera against H3N2 virus. (B) Western Blot analysis of E proteins in Sf9 cells applying mouse polyclonal sera against E protein. (C) Electron micrograph of negatively-stained chimeric VLPs E-HA2. Bar, 50 nm. (D) Indirect immunofluorescent assay (IFA) of E, HA and M1 proteins in Sf9 cells. E, HA, and M1 proteins were stained using anti-E (mouse), anti-HA (mouse), and anti-M1 (rabbit) primary antibodies. FITC-conjugated (anti-mouse) and Cy3-conjugated (anti-rabbit) secondary antibodies were used to visualize the localization of the proteins. 4′,6-diamidino-2-phenylindole (DAPI) was applied in cell nuclei staining. Bar, 50 nm.

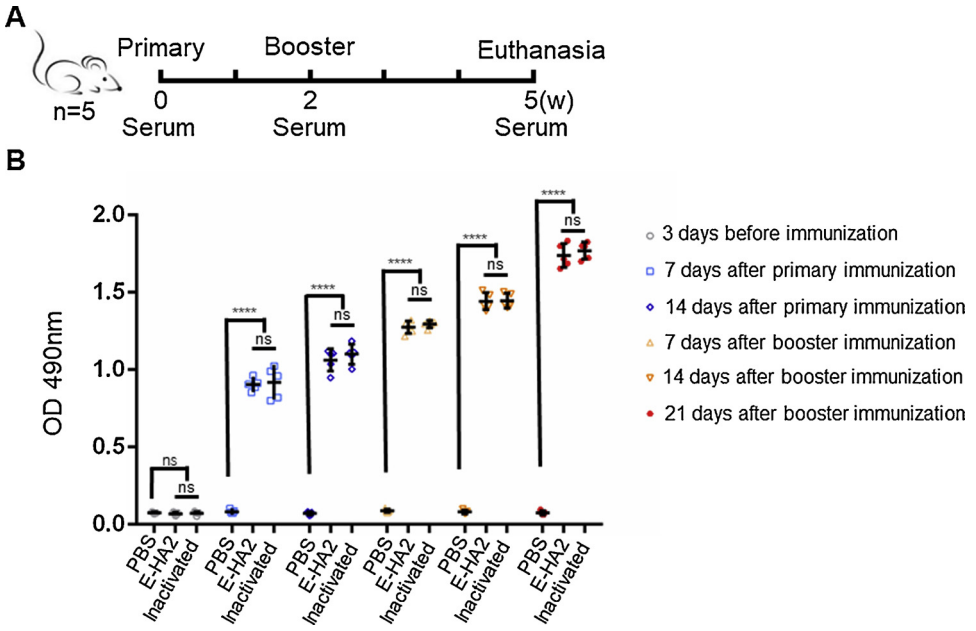

Fig. 3. Immunization of chimeric VLPs in mice. (A) Experimental schedule of mice immunized with chimeric VLPs E-HA2 (n = 5/group). (B) Antibody responses against E protein at different time points after immunization. **** indicates $P < 0.00001$.

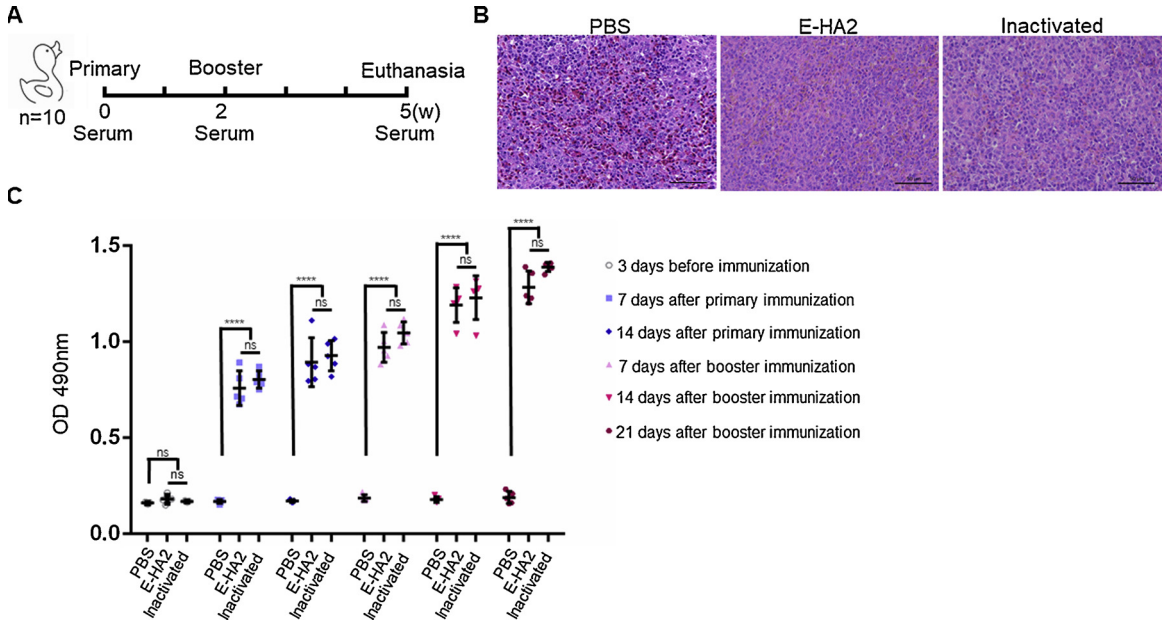

Fig. 4. Immunization of chimeric VLPs in mice. (A) Experimental schedule of mice immunized with chimeric VLPs E-HA2 (n = 10 per group). (B) HE-stained spleen sections of immunized groups. Bar, 50 μm. (C) Antibody responses against E protein at different time points after immunization. **** indicates $P < 0.00001$.

Table 2
Weight of each duck on the 14th day after challenge.

group	dose	duck weight after challenge(g)									
		1	2	3	4	5	6	7	8	9	10
E-HA2[a]	4 μg	1558	1625	1617	1573	1617	1677	1601	1704	1553	1586
Inactivated[b]	4 μg	1618	1594	1628	1631	1587	1579	1719	1579	1602	1655
PBS	0.5 m L	1586	1557	1528	1625	1584	1513	1480	1662	1581	1608

[a] Indicates P > 0.05.
[b] Indicates P < 0.05.

protein could provide complete protection against DTMUV challenge (Ma et al., 2016) and Goose Tembusu virus (GTV) challenge (Zhao et al., 2015a). DNA vaccine based on the E glycoprotein gene could stimulate high titers of the specific antibody to protect the ducks against DTMUV infection (Huang et al., 2018; Tang et al., 2018).

VLPs are self-assembled particles consisting of viral structural proteins only. The lack of viral genomic DNA or RNA constrains the particles from self-replication. VLP vaccine is a feasible alternative to live attenuated vaccines because they are noninfectious, highly immunogenic, and accessible to quality control. In flavivirus VLP vaccine development, co-expression of precursor membrane (prM) and envelope (E) is sufficient for the assembly and release of the VLPs, as in the cases of Japanese encephalitis virus (JEV) (Liu et al., 2014; Urakami et al., 2017), Dengue virus (DENV) (Yamaji and Konishi, 2016), and Zika virus (ZIKV) (Boigard et al., 2017).

In this study, we constructed a VLP containing recombinant DTMUV E, influenza virus H3N2 HA and M1 proteins. The co-localization of E-HA2 and M1 at the surface of the infected cells, as well as the automatically formed chimeric VLPs with a diameter of 80–100 nm, suggest similar morphological characteristics of the chimeric VLPs studied in the current research to the standard influenza VLPs and other chimeric VLPs based on H3N2 HA and M1 (Lv et al., 2014; Shen et al., 2013). The chimeric VLPs were then prepared quantitatively and used as antigens in BALB/c mice and female SPF ducks. When compared with the DTMUV inactivated strain, the level of E-specific antibody responses of chimeric VLPs groups was similar ($p > 0.05$) both in BALB/c mice and SPF ducks. However, considering the advantages of genetic engineering vaccines in biosafety, lack of immune suppressors and allergens, and convenience for mass production, chimeric VLPs applied in the current research still serve as a potential vaccine candidate. For instance, although both chimeric VLPs and inactivated strain could provide protection in ducks when challenged with homologous DTMUV strain, 10% of the ducks (1/10) immunized with inactivated strain showed DTMUV symptoms as reluctance to move. Furthermore, we also took advantage of the well-developed influenza VLPs platform to generate the chimeric E-HA2 VLPs in the current research. Taken together, the chimeric VLPs used in the current research showed the potential for use as a candidate vaccine against DTMUV.

Table 3
Number of ducks presenting clinical signs and virus shedding after challenge.

group	Anorexia	Diarrhea (1–4 dpc)	reluctance to move (1–4 dpc)	Viral shedding (5 dpc, Cloacal swabs)
E-HA2[a]	1/10	1/10	0/10	0/10
Inactivated[a]	1/10	1/10	1/10	0/10
PBS	6/10	5/10	6/10	9/10

[a] Indicates P < 0.05.

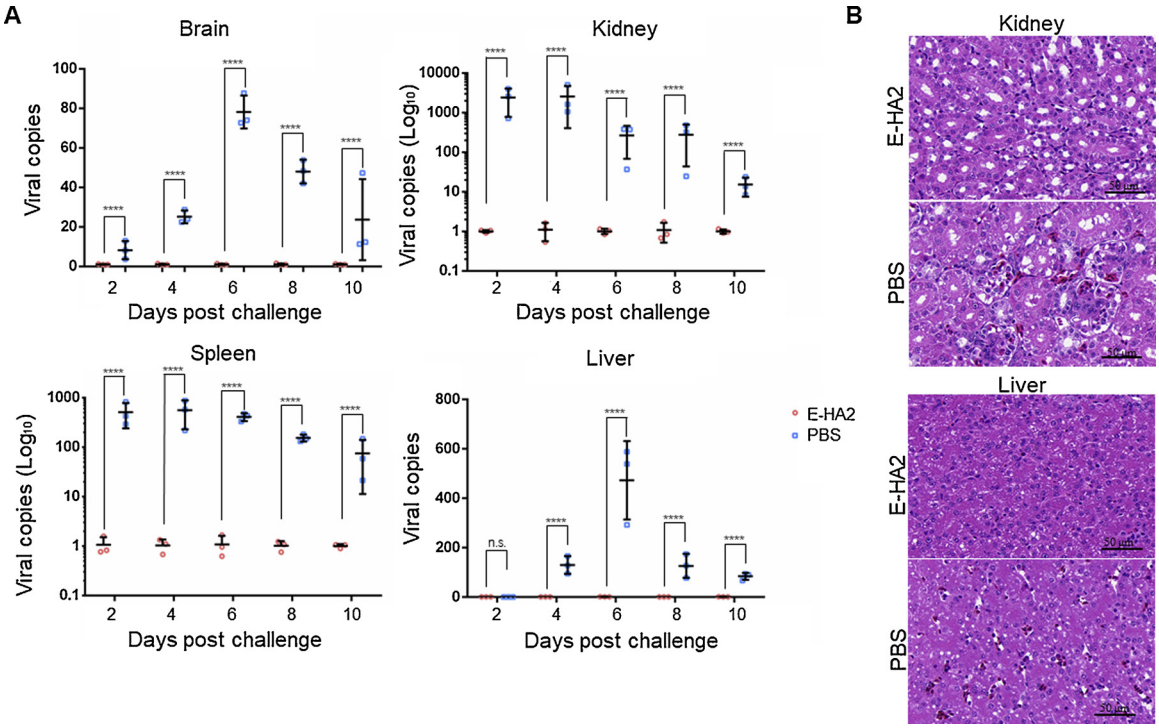

Fig. 5. Viral loads and histopathological changes in different organs. (A) Viral loads in livers, brains, spleens, and kidneys of the E-HA2 vaccinated and control group. (B) HE-stained liver and kidney sections of the E-HA2 vaccinated and control groups. Bar, 50 μm.

It was reported a trivalent vaccine based on an attenuated duck enteritis virus (DEV) strain containing the HA gene of H5N1, PrM and E gene of DTMUV could enhance both humoral and cell-mediated immune responses to H5N1 and DTMUV. Furthermore, the trivalent vaccine candidate could protect ducks against virulent H5N1, DTMUV, and DEV challenges (Zou et al., 2017). The HA stalk region has attracted researchers as a target for the development of universal vaccines, while the HA2 subunit of the influenza virus makes up the major part of the HA stalk region and is highly conserved within subtypes (Fouchier et al., 2005; Raymond et al., 2018; Zhang et al., 2019). In the current study, we constructed this chimeric VLPs containing the antigenic proteins of both influenza virus (HA2) and DTMUV (E), though the protection efficacy of the chimeric VLPs to influenza virus were not evaluated in the current study, further research is required to explore whether the chimeric VLPs used in the current study could protect ducks from influenza virus infection. Furthermore, it is also worth applying biochemistry and structural biology method in order to understand whether replacement of transmembrane domain would later the structure of the E protein thus modified the immunogenicity of the protein.

5. Conclusion

In summary, in the current study, we constructed chimeric VLPs containing DTMUV E protein and influenza HA2 subunit. Our data presented that the chimeric VLPs could induce antibodies against DTMUV E protein in both mice and ducks. Furthermore, our data of efficiency test in ducks showed the VLPs could protect animals from homologous virus challenge. Taken together, the E-HA2 VLPs may be considered as a vaccine candidate and may serve as a bivalent vaccine candidate and provide directions for future vaccine development.

Author's contributions

M.S. and Z.X. designed the experiments. L.L., Y.Z., J.D., J.Z., C.Z., and J.Q. carried out the experiments. L.L. and Y.Z. wrote the manuscript and prepared the figures. All authors read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.108425.

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