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Recombinant Newcastle disease virus (NDV) expressing Duck Tembusu virus (DTMUV) pre-membrane and envelope proteins protects ducks against DTMUV and NDV challenge

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ABSTRACT

The newly emerged Duck Tembusu virus (DTMUV) is responsible for considerable economic loss in waterfowl-raising areas in China since 2010. Meanwhile, the virulent Newcastle disease virus (NDV) has also caused sporadic outbreaks in waterfowl. The individual vaccines against both diseases are available, however, there is no bivalent or combined vaccine for either disease. Here, we constructed a recombinant NDV-vectored vaccine candidate that expresses the pre-membrane (prM) and envelope (E) genes from DTMUV, designated as aGM/prM + E. The foreign prM and E proteins were stably expressed in aGM/prM + E and exhibited similar pathogenicity but higher growth kinetics than those of the parental virus. The aGM/prM + E carries a fusion cleavage site in accordance with avirulent viruses that have been frequently isolated from waterfowl, and induced remarkably ($p < 0.001$) higher NDV-specific hemagglutination inhibition (HI) titers than commercially available live NDV vaccines (LaSota strain). The aGM/prM + E also elicited significantly higher ($p < 0.05$) virus neutralization (VN) titers than commercially available DTMUV inactivated vaccines (HB strain). The aGM/prM + E not only provided complete protection against NDV challenge but also reduced the gross lesions on ovarian folliculi and provided 80% protection against DTMUV in ducks. We note that the aGM/prM + E vaccine can prevent challenged ducks from shedding of NDV and DTMUV. Our results suggest that the candidate vaccine aGM/prM + E would help decrease NDV and DTMUV transmissions in waterfowl raising areas in China.

1. Introduction

The newly emerging Duck Tembusu virus (DTMUV) causes sudden egg-dropping symptoms in layer ducks and was first reported in China in April 2010 (Su et al., 2011; Yan et al., 2011). Later, infections and deaths were reported in chickens (Chen et al., 2014b), ducklings (Lu et al., 2016), geese (Ti et al., 2015), pigeons (Dai et al., 2015), and sparrows (Tang et al., 2013). Infected layer ducks typically exhibit a considerable decrease in egg production; primary pathologic changes include ovaritis, ovarian hemorrhage, ovarian atrophy and rupture (Wang et al., 2015b). In broiler ducks and geese, the primary clinical signs include anorexia, depression, retarded growth, greenish diarrhea, ataxia, reluctance to walk, progressive paralysis and grossly swollen spleens (Yun et al., 2012). Although commercial vaccines have been developed, the outbreaks of this severe contagious disease among domestic fowl have resulted in large economic losses for the poultry industries in China and Southeast Asia (Homonnay et al., 2014; Thontiravong et al., 2015; Lu et al., 2016).

Duck Tembusu virus is a mosquito-borne *Ntaya* group flavivirus from the family *Flaviviridae*. It is a spherical and enveloped virus, approximately 40–60 nm in diameter. The genome consists of single-stranded, positive-sense RNA, approximately 11 kb in length with a long open reading frame (ORF) that encodes a large polyprotein (Su et al., 2011; Yan et al., 2011). The polyprotein is cleaved by viral and cellular proteases into three structural proteins (Capsid, C; pre-membrane/membrane, prM/M; and envelope, E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Su et al., 2011; Yan et al., 2011). Among these, E protein is the major virion surface protein and the primary target for neutralizing antibodies (Heinz, 1986), and therefore, the first choice for subunit vaccine

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development (Ma et al., 2016; Zhao et al., 2015). However, the glycosylated E protein and the nonglycosylated M protein of flaviviruses are both associated with the lipid envelope (Heinz, 1986). Moreover, it has been shown that the chaperone-like activity of prM is necessary for the proper folding of E in Tick-borne encephalitis virus (TBEV) (Konishi and Mason, 1993; Lorenz et al., 2002). Hence, expressing both prM and E protein represents a more thorough, promising approach towards vaccine development.

After massive vaccination campaigns, Newcastle disease (ND) has been well controlled in China. However, sporadic reports of Newcastle disease virus (NDV), especially in waterfowl, still raise concerns that non-immune waterfowl may play important roles in the dispersal of NDV (Liu et al., 2003; Liu et al., 2008). Waterfowl are reservoirs of NDV and deaths have been reported (Higgins, 1971), some of which were caused by a virus of the genotype VII (Jinding et al., 2005; Qin et al., 2008), a predominant genotype in recent years in China (Zhang et al., 2014). As documented in the literature (Hu et al., 2009; Xiao et al., 2012; Liu et al., 2015), there are many advantages to developing a genotype-matching recombinant vaccine against the currently predominant NDV. Concerning the dispersal of NDV and the outbreaks of DTMUV in waterfowl in China, the development of a vaccine effective against both NDV and DTMUV is urgently needed.

An NDV vector expressing foreign proteins can elicit broad, protective immune responses including humoral and cellular immunity, as well as systemic and mucosal immunity, and its safety in many animal models has been demonstrated (Huang et al., 2003; Ge et al., 2011; Duan et al., 2015). It has also been shown that recombinant NDV can serve as a highly effective vaccine vector for protection against other pathogens (Huang et al., 2004; DiNapoli et al., 2007; Veits et al., 2008; DiNapoli et al., 2010; Kanabagatte Basavarajappa et al., 2014; Khattar et al., 2015; Kim et al., 2015; Hu et al., 2017). Naturally occurring avirulent NDVs are routinely used as live vaccines throughout the world (Huang et al., 2004), however, a genotype matched vaccine can provide better protection against currently circulating strain of NDV genotype VII (Hu et al., 2009; Xiao et al., 2012; Liu et al., 2015; Sun et al., 2017). Meanwhile, the commercial inactivated DTMUV vaccines clinically provide partial or no protection because some ducks was infected as early as 1–2 weeks of age and developed severe clinical signs even death (Li et al., 2015). Leveraging the advantages of the NDV vector, the development of a bivalent recombinant vaccine candidate that expresses both prM and E protein of DTMUV would be helpful for controlling the transmission of both NDV and DTMUV.

In this paper, we describe a recombinant attenuated NDV (aGM) expressing the host-protective immunogen prM and E from the DTMUV strain JM. The recombinant aGM/prM + E virus stably maintained and expressed the prM and E genes and the growth kenetics were higher than those of the parental virus. Vaccination with the recombinant aGM/ prM + E induced strong humoral immune responses against both NDV and DTMUV, providing complete protection against virulent NDV and 80% protection against DTMUV after boosting. These results clearly suggest that it is possible to develop a commercially viable, bivalent recombinant vaccine that provides protection against both of these economically draining diseases.

2. Materials and methods

2.1. Ethics statement

This study was carried out 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 the People's Republic of China. The animal experiments with virulent NDV were performed in Animal Biosafety Level 3 facilities. All animal experiments were conducted under the guidance of South China Agricultural University's Institutional Animal Care and Use Committee, Institute of Animal Health, Guangdong Academy of Agricultural Sciences Experimental Animal Welfare Ethics Committee, and the Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The protocol was reviewed and approved by the Committee on the Ethics of Animal Experiments of the Animal Biosafety Level 3 Committee of South China Agricultural University and the Committee on the Ethics of Animal Experiments of Institute of Animal Health, Guangdong Academy of Agricultural Sciences Experimental Animal Welfare Ethics Committee. The approve ID is 2015-005.

2.2. Viruses, plasmids, and cells

Duck Tembusu virus JM (GenBank accession number: JN811559) was isolated from layer ducks in 2011 (Li et al., 2012). The plasmid containing the virulent NDV cDNA clone, TVT-rGM, was generated by five fragments that sequentially inserted into the backbones by the unique restriction enzyme sites (*Age* I, *Asc* I, *Spe* I and *Mlu* I) they shared as described in the literature (Sun et al., 2017). BSR T7/5 cells, stably expressing the T7 phage RNA polymerase, were kindly provided by Dr. Zhigao Bu (Harbin Veterinary Institute, Harbin, China).

2.3. Attenuation of virulent NDV rGM

To construct the cDNA clone of attenuated rGM, the nucleotide sequences of the cleavage site were modified from 5′-AGGAGACAAAA ACGCTTT-3′ to 5′- gaGAGACAggAACGCcTT-3′ by fusion PCR mutagenesis, which correspondingly changed the motif from ^{112} RRQKRF ^{117} to ^{112} ERQERL ^{117} (Fig. 1). First, two products were generated by P2-2879U/FCls-I-Lower and F-Cls-I-Upper/P2-6767L primer sets (Table 1), respectively. Secondly, primers P2-2879U and P2-6767L (Table 1) were used to generate the mutated form using the two products noted above as templates and then cloned into the cDNA clone backbone using *Age* I and *Asc* I restriction enzyme sites (for cDNA cloning details, see Sun et al., 2017). Similarly, a *Pme* I restriction enzyme site was introduced into the inter-region of gene P and M by changing GCTTCAAC to GtTTaAAC by P2-2879U/Pme-6193L and Pme-6200U/P2-6767L primer sets (Table 1). The attenuated full-length cDNA clone, designated aGM, was confirmed by sequencing. Virus recovery was performed as described in the literature (Sun et al., 2017).

2.4. Construction and virus recovery of aGM/prM + E

To insert the foreign genes, the prM and E genes of the DTMUV JM strain were flanked with the GS (gene start) and GE (gene end) sequences of NDV harbored in the inter-region sequences of the P and M genes (Fig. 1). Given the lack of initiation and stop codons for prM and E, ATG and TAA were added to make an ORF. Additionally, a Kozak sequence was inserted upstream of the ATG. To introduce foreign genes, the primers JM-prM/E-U and JM-prM/E-R were used (Table 1). Then, the products were cloned into pMD18-T vector (Takara, Dalian, China) and digested by *Pme* I restriction enzyme. The fragment containing prM and E genes was subcloned using the *Pme* I restriction enzyme site of the aGM cDNA clone, resulting in the plasmid designated as aGM/prM + E. After sequencing, virus recovery was then performed (Sun et al., 2017). The rescued virus was identified by RT-PCR with primers P2-2879U and P-3330L (Table 1) to confirm the insertion of prM and E genes.

2.5. The pathogenicity and viral growth kinetics of aGM/prM + E

The aGM/prM + E fresh allantoic fluids were evaluated for pathogenicity by mean death time (MDT) and intracerebral pathogenicity index (ICPI) (Roohani et al., 2015). And 7 day-old ducks were nasally inoculate with $10^{8.0}$ EID_{50} (50% egg infections dose) aGM/prM + E recombinant virus, and passaged three times in ducks. The EID_{50} of the virus and the viral growth kinetics were determined as described in the literature (Sun et al., 2017). Briefly, primary chicken embryo fibroblasts

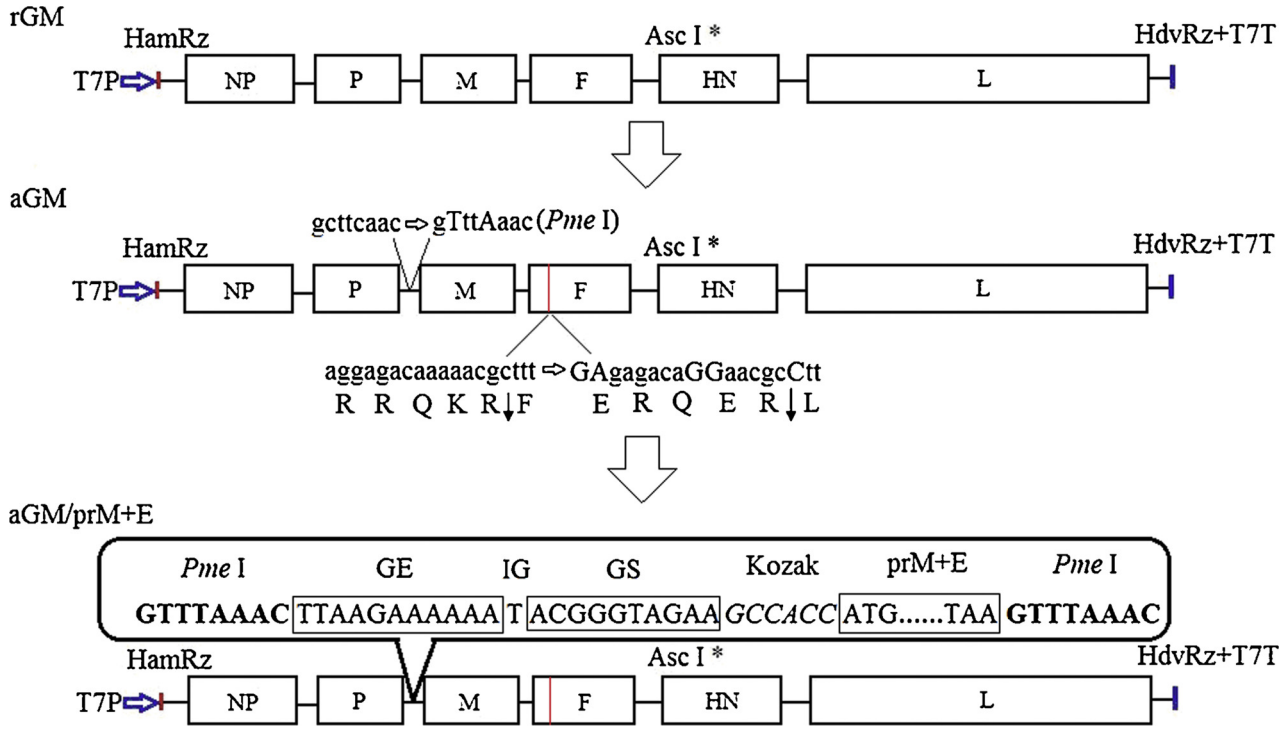

Fig. 1. Simplified schematic for the construction of the full-length aGM/prM + E cDNA. Briefly, the cleavage site of rGM was mutated by fusion PCR for attenuation and the unique *Pme* I restriction enzyme site was introduced simultaneously to enable foreign gene insertion between the P and M genes. Then the prM and E genes with gene end (GE), intergenic (IG), gene start (GS) and Kozak sequences were inserted using the *Pme* I restriction enzyme site. The HamRz sequence is denoted by a red vertical line. The *Asc* I restriction enzyme site is labeled with an asterisk as a biomarker. The blue arrow points to the T7 promoter (T7P) in the vector, HdvRz and T7 terminator (T7T) are identified by the blue vertical line. The red vertical line in the F gene indicates the avirulent cleavage site. The mutated nucleotides are capitalized, the *Pme* I restriction enzyme site is bolded, the GE, GS, prM, and E sequences are boxed, and the Kozak sequences are italicized. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

(CEFs) were infected with aGM and aGM/prM + E with or without TPCK-treated trypsin (1 μg/mL) at a multiplicity of infection (MOI) of 1 or 0.01. The infected cultures were harvested at 12, 24, 36, 48, 60, and 72 hours post-infection (hpi), and the viral titers were determined in triplicate using the standard median tissue culture infective dose ($TCID_{50}$) assay. Viral titers were presented using GraphPad Prism 5 (GraphPad, California, USA) and studied by two way ANOVA multiple comparisons statistical analysis.

2.6. Transmission electron microscopy

The allonatic fluid of aGM/prM + E was purified by sucrose gradient ultracentrifugation and added to the copper grid. It then underwent negative staining with phosphotungstic acid (2.5%, pH 7.0). Then, images were collected using the H-7650 electron microscope (Hitachi, Tokyo, Japan).

2.7. Western blot

The aGM and aGM/prM + E virions was purified by sucrose gradient ultracentrifugation, and the culture medium of aGM and aGM/prM + E were collected at 48 hpi respectively by infected the CEFs at a MOI of 1. Then, the virions and culture medium were separated by SDS-PAGE and electro-transferred to 0.22 μm PVDF membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween-20 (TBST) for 1 h at 37 °C and then incubated for overnight at 4 °C with mouse anti-NDV polyclonal antibodies and rabbit anti-DTMUV polyclonal antibodies that prepared using the sucrose gradient ultracentrifugation purified viruses as antigen. Membranes were washed in TBST and incubated with IRDye® 800CW goat anti-

Table 1
Primers for the mutation and cloning of NDV and DTMUV.

Name	Primer sequences (5′ → 3′)[a]
P2-2879U	TTGACCGGTGCAGCACCCCTCTGAAT
F-Cls-I-Lower	CTAT**AAgGCGTTccTGTCTCtc**TCCTCCAGACATGGACACAGACCCTT
F-Cls-I-Upper	GGAGGA**gaGAGACAggAACGCcTT**ATAGGTGCTGTTATTGGCAGTG
P2-6767L	GTTGGCGCGCCACATTCGCTATTGTTCTCAG
Pme-6200U	AATCCACGtTTaAACACCCCATACAACAGCCCTCT
Pme-6193L	TGTTGTATGGGGTGtTTaAACGTGGATTTGCAAGG
JM-prM/E-U	GTTTAAACTTAAGAAAAAATACGGGTAGAA*GCCACC***atgctgaagcttggaaattat**
JM-prM/E-R	GTTTAAAC**ttaggcattgacatttactgccaggaag**
P-3330L	GGCTGCTGGAAGGGAGGGCAGAAT

[a] The primer sequences matching NDV are capitalized and those matched to DTMUV are lowercased and bolded. The cleavage site of NDV is bolded and the mutated nucleotide is shown in lowercase. Restriction enzyme sites are underlined and the kozak sequence is italicized.

rabbit and anti-mouse IgG (1:10,000) respectively for 1 h. At last, membranes were washed and visualized using the Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, USA).

2.8. Immunofluorescence assays

For immunofluorescence analysis, CEFs was plated on coverslips in 35 mm dishes and infected with aGM/prM + E or aGM, JM alone. After 24 h, the cells were fixed with ice-cold ethanol for 15 min at room temperature and blocked in phosphate buffer saline (PBS) containing 10% (w/v) milk at 4 °C for 16 h. After three washes, the cells were incubated with anti-NDV rabbit polyclonal antibodies and anti-DTMUV mouse polyclonal antibodies for 1 h at room temperature. After being washed three times with PBS containing 0.1% Tween 20, the cells were stained with DyLight 549 AffiniPure goat anti-rabbit antibodies (Earthox, California, USA) and DyLight 488 AffiniPure goat anti-mouse antibodies (Earthox, California, USA) for 30 min. Finally, the cells were washed three times with PBS, stained with DAPI, and analyzed using a confocal laser microscope (LSM 710, Zeiss, Oberkochen, Germany).

2.9. Protection studies of aGM/prM + E recombinant virus in layer ducks

Six groups of 150-day-old layer ducks (NDV and DTMUV antibody-free, N = 60, n = 10) were raised in separate compartments. Among them, three groups were used for NDV challenge-protection study, another three groups were used for DTMUV challenge-protection study. In the first three groups, $10^{6.0}$ EID_{50} of fresh aGM/prM + E allantoic fluid, a dose of commercial live LaSota vaccine (Winsun, Guangdong, China) or PBS were immunized subcutaneously respectively. The rest three groups were immunized either with $10^{6.0}$ EID_{50} of fresh aGM/prM + E allantoic fluid or commercial oil emulsion DTMUV vaccine (HB strain) (Rinpu, Tianjin, China) or PBS. Serum samples were collected at 7 and 14 days post immunization (dpi) and measured with a hemagglutination inhibition (HI) test and virus neutralization (VN) assay. Meanwhile, all of the ducks in the vaccinated groups received the same dosage of the second immunization for boosting. Serum samples were also collected at 7 and 14 dpi for HI and VN measurement after boosting. At 14 days after the second immunization, the first three groups were challenged intranasally with $10^{5.0}$ ELD_{50} of NDV (GM strain). Oropharyngeal and cloacal swabs were collected at 1, 3 and 5 days post challenge (dpc) for virus isolation. The remaining three groups were challenged subcutaneously with $10^{3.0}$ ELD_{50} of DTMUV (JM strain). Oropharyngeal and cloacal swabs were collected at 1 and 3 dpc for virus isolation. At 4 dpc, the ducks were euthanized, and the ovarian folliculi were examined for gross lesions.

3. Results

3.1. Attenuation of virulent NDV rGM and construction of aGM/prM + E

Due to the presence of only one large ORF existing in the genome of DTMUV, the prM and E genes were cloned together as one ORF by the addition of ATG and TAA codons (Fig. 1). Simultaneously, the GE and GS sequences were flanked to bring the foreign sequences under the control of transcriptional regulation sequences (DiNapoli et al., 2007; Ge et al., 2011; Kim et al., 2015) and match the “rule of six” genetic feature of NDV (Kolakofsky et al., 2005). As expected, the rescued virus aGM shifted the cleavage site from ^{112} RRQKRF ^{117} to ^{112} ERQERL ^{117} (Fig. 2A), as well as a unique restriction enzyme site in the inter-region between the P and M genes (Fig. 2B). By RT-PCR confirming, the products of the rescued virus aGM/prM + E was in the size range of 2000–3000 bp (expected amplicon size of 2516 bp) and aGM was about 500 bp (expected amplicon size of 478 bp) (Fig. 2C).

3.2. Recovery and biological properties of the recombinant virus aGM/prM + E

The recombinant virus was recovered by cotransfection of plasmids aGM/prM + E, pCI-NP, pCI-P, and pCI-L, and passaged once on specific-pathogen-free (SPF) embryonated eggs. The aGM and aGM/prM + E viruses had ICPI values of 0.5 and 0.4, and MDT values of 106 h and 116 h respectively, indicating that both viruses were avirulent. After three passages in ducks, no clinical syndromes were observed. Then, RT-PCR was used for cleavage site conforming, the results showed no reversion happened. The viral titers of aGM and aGM/prM + E were $10^{9.0}$ EID_{50}/mL and $10^{8.7}$ EID_{50}/mL respectively. Single-growth kinetics of the recombinant virus aGM/prM + E on CEF cells revealed that the viral titers with trypsin were slightly higher than those without trypsin at 12–60 h. It was about 10-fold higher than the parental virus aGM with trypsin. However, the peak of aGM/prM + E and aGM viruses were both at 60 h with trypsin and 48 h without trypsin (Fig. 3A). The viral titers of aGM/prM + E were significant higher ($p < 0.05$) than those of aGM at 24 h with trypsin and at 12, 36, 48, 60 and 72 h without trypsin by two way ANOVA multiple comparisons statistical analysis. As to multiple-growth kinetics, results revealed that the titers of aGM/prM + E increased rapidly at 24 h, and the viral titer with TPCK-treated trypsin at this time was significant higher ($p < 0.05$) than that without TPCK-treated trypsin. The viral titers peaked at 60 and 48 h with or without trypsin respectively (Fig. 3B), similar to results from the single-growth kinetics. However, the peak of parental virus aGM was 48 h with or without trypsin. The viral titers of aGM/prM + E were significant higher ($p < 0.05$) than those of aGM at 24 h with trypsin and at 24, 60 and 72 h without trypsin. The highest titer from the single-growth kinetics was approximately 10-fold higher than that from the multiple-growth kinetics for both aGM/prM + E and aGM (Fig. 3B). The viral titers from both growth kinetics revealed that the replication of the recombinant virus aGM/prM + E, but not the aGM virus, was not dependent on exogenous trypsin.

3.3. Expression of prM and E proteins by recombinant virus aGM/prM + E

To examine the viral morphology of aGM/prM + E, the parental virus aGM and aGM/prM + E underwent ultracentrifugation and were imaged using transmission electron microscopy. The virions of aGM/prM + E were comparable with aGM (Fig. 4A and B), indicating the insertion of foreign prM and E protein did not affect the morphology of NDV. Then, the harvests of aGM and aGM/prM + E that purified by sucrose gradient ultracentrifugation and the infected cell culture medium of CEFs were seperated by SDS-PAGE, and underwent western blot. The prM and E protein can be detected in the purified virions (Fig. 4C) and the culture medium (Fig. 4D) of aGM/prM + E, indicating the integration of prM and E protein in the envelope. To further examine the expression of the foreign proteins, indirect confocal immunofluorescence staining assay was used. When examined the aGM/prM + E virus infected CEFs with anti-NDV and anti-DTMUV antibodies, both green and red fluorescence was observed. After merging both fluorescent images, green and red fluorescence co-localized to the same cells, confirming the foreign protein is co-expressed with the NDV proteins of the recombinant virus in the infected cells (Fig. 5). While the aGM and JM viruses infected cells were only positive for immunostaining of NDV or DTMUV serum respectively (Fig. 5).

3.4. Immunogenicity in layer ducks

The mean HI titer of the aGM/prM + E-vaccinated group reached 5.6 $\log_2$ at 14 dpi, which was remarkably higher than the LaSota vaccine ($p < 0.001$, Fig. 6A) by two way ANOVA multiple comparisons statistical analysis. After boosting, the mean HI titers of aGM/prM + E virus were higher than 7.0 $\log_2$, and still remarkably higher than the LaSota vaccine ($p < 0.001$, Fig. 6A). All of the ducks in the three

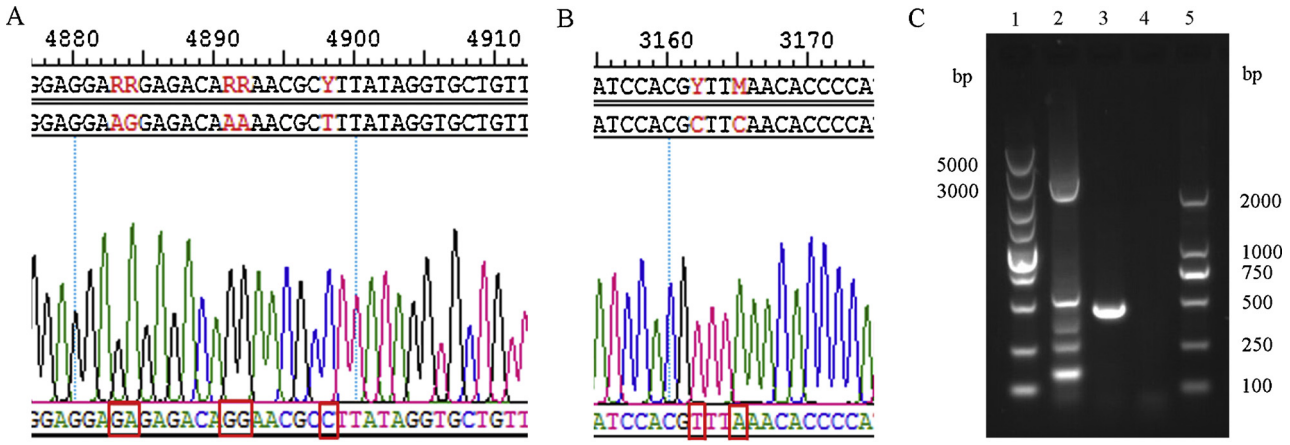

Fig. 2. Mutated nucleotides of aGM and identification of foreign gene insertion of aGM/prM + E. (A) Modification of the nucleotides of the F protein cleavage site. The mutated nucleotides are boxed in red. (B) The unique *Pme* I restriction enzyme site, generated by C3162T and C3165A mutation, are boxed in red. (C) RT-PCR ampification of aGM/prM + E and aGM. 1: DL 5000 DNA marker; 2: RT-PCR result of aGM/prM + E, the expected amplicon size are 2516 bp; 3: RT-PCR result of aGM, the expected amplicon size are 478 bp; 4: Negative control; 5: DL 2000 DNA marker (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

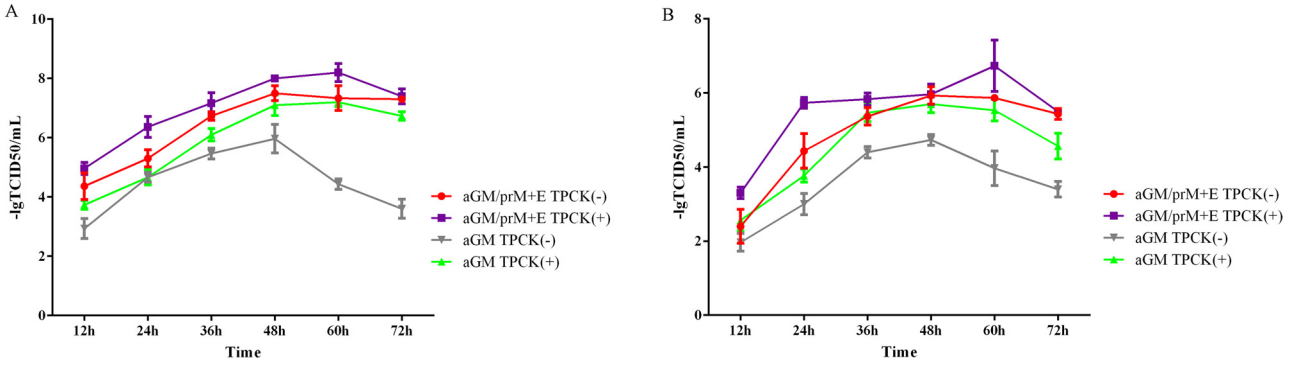

Fig. 3. Growth kinetics of the recombinant aGM/prM + E and aGM virus on CEF cells. (A) Single growth kinetics of aGM/prM + E and aGM. CEF cells were infected by aGM/prM + E and aGM with or without 1 μg/mL TPCK-treated trypsin at a multiplicity of infection of 1. The infected cultures were harvested at 12, 24, 36, 48, 60, and 72 hours post-infection (hpi). The viral titers were calculated in triplicate using a standard median tissue culture infective dose ($TCID_{50}$) assay. (B) Multiple growth kinetics of aGM/prM + E and aGM. CEF cells were infected by aGM/prM + E and aGM with or without 1 μg/mL TPCK-treated trypsin at a multiplicity of infection of 0.01. The infected cultures were harvested at 12, 24, 36, 48, 60, and 72 hpi. The viral titers were determined in triplicate with a standard $TCID_{50}$ assay. The significance of viral titers of aGM/prM + E and aGM with or without TPCK-treated trypsin were compared with two way ANOVA multiple comparisons statistical analysis.

groups challenged with virulent NDV (GM strain) exhibited no clinical signs of disease, however, the viral shedding results varied. As seen by the data in Table 2, the aGM/prM + E-vaccinated group exhibited no viral shedding after challenge, and both the LaSota-vaccinated and PBS-inoculated groups shed the virus at 1 and 3 dpc. However, the LaSota-vaccinated group had 20% viral shedding in oropharyngeal swabs at 3 dpc, and the PBS-inoculated groups had 50% viral shedding in oropharyngeal swabs and 20% viral shedding in cloacal swabs at the same time point. None of the ducks had evidence of viral shedding by 5 dpc.

The mean VN titer that aGM/prM + E elicited reached 1.9 log_2 at 14 dpi, which was remarkably higher than that of the DTMUV inactivated vaccine ($p < 0.001$, Fig. 6B). And it was significantly higher than that of the DTMUV inactivated vaccine at 21 dpi after boosting ($p < 0.05$, Fig. 6B). Although there were no significant differences between the mean VN titers (4.0 log_2) of the aGM/prM + E-vaccinated group and the DTMUV-inactivated vaccine-inoculated group at 28 dpi, the gross lesions on the ovarian folliculi revealed that two of the 10 ducks in the aGM/prM + E-vaccinated group had clinical signs of DTMUV infection, and four of the 10 ducks in the HB-vaccinated group exhibited typical lesions including ovarian hemorrhage, ovaritis, and regression (Table 2). The viral shedding results showed that aGM/prM + E-vaccinated group had 40% viral shedding in cloacal swabs at 3 dpc, while HB-vaccinated and PBS-inoculated group had 60% and 80% viral shedding respectively at the same time point. The results of viral shedding in the DTMUV challenged groups at 1 dpc together with the oropharyngeal swabs at 3 dpc were 0–30%, indicating low sensitivity of virus isolation by oropharyngeal swabs or at 1 dpc. It is of note that the VN titer of individual ducks higher than 4.0 log_2 can provide full gross lesions protection against DTMUV challenge. As expected, ducks in the PBS mock-immunized group all exhibited greenish diarrhea at 2 dpc as well as typical lesions on their ovarian folliculi at 4 dpc.

4. Discussion

DTMUV and NDV have caused significant economical loss in the waterfowl-raising areas in China. Vaccines remain the most cost-effective strategy to control these pathogens. However, the available oil-emulsified, inactivated vaccines usually take six weeks to confer immune protection (Lin et al., 2015; Zhang et al., 2017), and two or three doses are required for adequate protection (Halstead and Thomas, 2011). Moreover, they can cause severe adverse reactions, including local inflammation and “egg drop” (Zou et al., 2017). There are also safety concerns about the possibility of reversion for the live attenuated vaccine against flavivirus (Jennings et al., 1994) and recombination

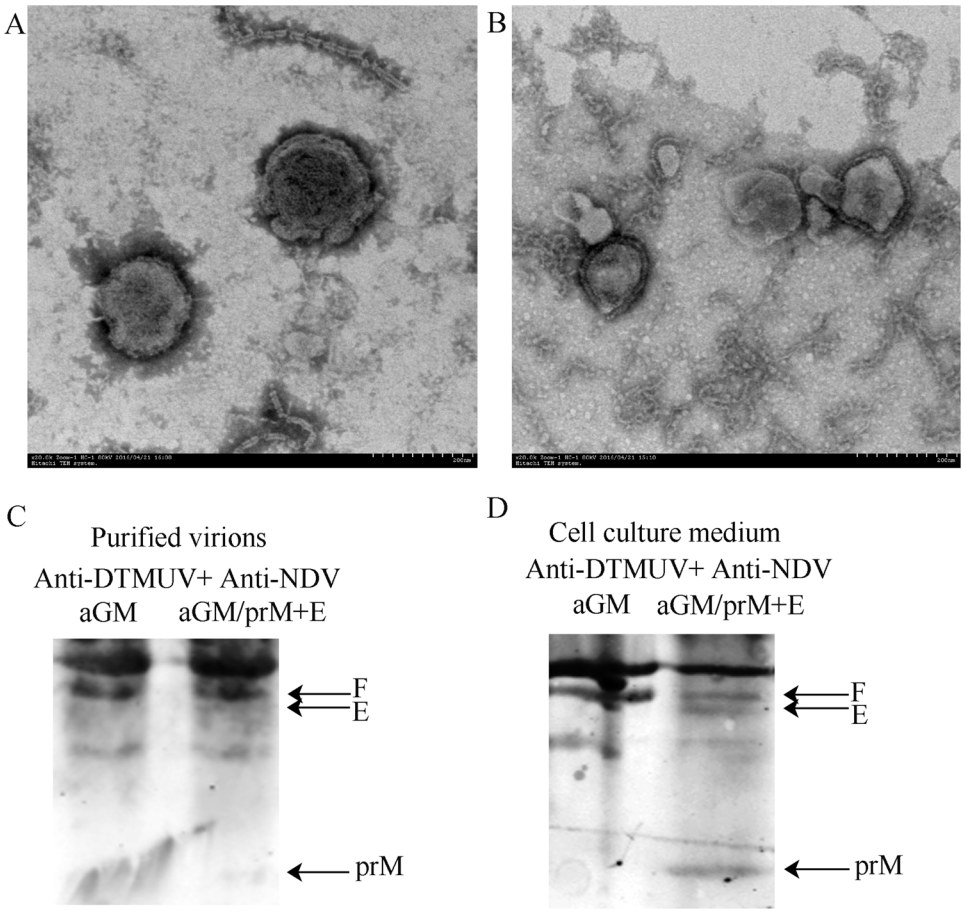

Fig. 4. Analysis of the recovered viruses by electron microscopy. (A) The aGM virions were negatively stained and observed by electron microscopy, scale bars (200 nm) are at the bottom right corner. (B) The aGM/prM + E virions were negatively stained and observed by electron microscopy. (C) The aGM and aGM/prM + E virions were purified by sucrose gradient ultracentrifugation and (D) the culture medium of aGM and aGM/prM + E on CEFs were separated by SDS-PAGE, transferred to 0.22 μm PVDF membranes and underwent Western blot. F stands for the fusion protein of NDV, E and prM stands for envelope protein and pre-membrane protein of DTMUV.

between the vaccine and other wild-type flaviviruses (Seligman and Gould, 2004).

In regards to NDV live vaccines, genotype-matched versions provided better protection in terms of eliciting higher HI titers and reducing viral shedding. The NDV vector can provide an alternative live vaccine platform for newly emerging viruses and non-cultivable pathogens; examples include expressing the S protein of SARS-CoV (DiNapoli et al., 2007), the glycoprotein (GP) of the Ebola virus (Wen et al., 2013), the hemagglutinin of H7N9 avian influenza virus (Hu et al., 2017), and the capsid and VP2 protein of Norwalk virus (Kim et al., 2015). Also, a recombinant vaccine has been developed against GPV in goslings (Wang et al., 2015a). In line with the advantages of NDV-vectored vaccines mentioned above, the development of a recombinant vaccine expressing the DTMUV foreign proteins to target newly emerging pathogens is feasible.

A previous study showed that an NDV vector from a virulent backbone was more efficient at inducing humoral, cellular, and mucosal immunity than the LaSota vector (Kim et al., 2014). So it would be more practical to use the predominant genotype VII virus as backbones. Additionally, as reported in the literature (Kim et al., 2007; Mase and Kanehira, 2015; Zhu et al., 2014), waterfowl represent reservoirs of NDV Class I and have been shown to be avirulent. Therefore, based on a recombinant NDV genotype VII (Sun et al., 2017), we mutated the cleavage site to match the feature of NDV Class I. During the insertion of foreign sequences, a plasmid with a single E gene insertion (designated aGM/E) was also constructed (data not shown). However, a viable virus could be only recovered from aGM/prM + E. Since secreted E without the prM protein of DTMUV can be expressed by a recombinant duck enteritis virus (Chen et al., 2014a), a truncated E gene might be necessary for foreign expression. Moreover, our results confirmed that prM is important for protection efficiency, further suggesting that prM is necessary for DTMUV vaccine development (Chen et al., 2014a). Another report (Zou et al., 2017) showed that recombinant duck enteritis virus expressing the HA protein of H5N1 plus prM and E protein of DTMUV conferred complete protection against a virulent DTMUV challenge, which re-emphasizes the importance of prM in vaccine protection efficacy. Although the aGM/prM + E virus carries foreign proteins, the virion shape was similar to that of the parental aGM virus. Furthermore, the expression of foreign prM and E proteins was confirmed by western blot, and the co-localization of NDV proteins and foreign DTMUV proteins around the cell membrane using confocal imaging of immunofluorescence staining, confirming correct expression patterns.

Insertion of foreign genes between the M and F genes generally leads to a decrease in pathogenicity, followed by longer MDT and decreased ICPI values (Nakaya et al., 2001). However, the insertion of foreign genes between the P and M proteins has little to no influence on the viral titers (Krishnamurthy et al., 2000; Engel-Herbert et al., 2003). As expected, the insertion of the prM and E genes increased MDT values from 106 h to 116 h, and the ICPI values decreased from 0.5 to 0.4, and there were no clinical syndromes observed after three passages on ducks. Moreover, the recombinant virus showed vigorous growth curves compared to the parental virus aGM with trypsin, especially without trypsin at 48 h or later. Our results showed that the aGM/prM + E virus does not depend on exogenously added trypsin for its propagation, which differed from the parental virus and our previous findings (Sun et al., 2017). The discrepancy might be attributed to the fusion activities of the foreign E protein of flavivirus (Lindenbach and Rice, 2003) because the HA titers of the recombinant virus were higher at pH 5.0 than those at pH 7.2 at 48 hpi (data not shown).

In order to evaluate the safety of the aGM/prM + E virus, the appetites and clinical responses of the layer ducks after immunization

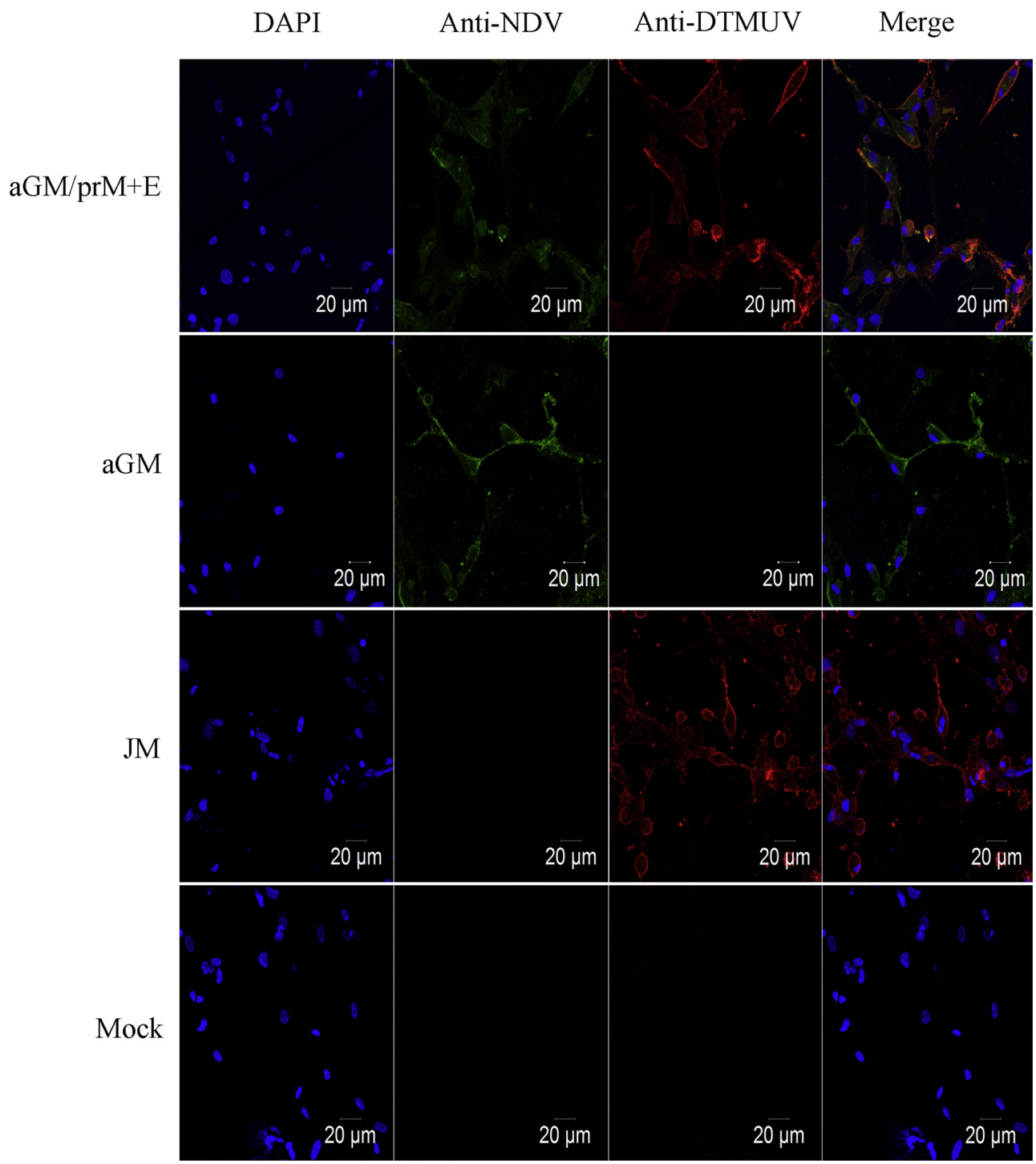

Fig. 5. Immunofluorescence analysis of prM and E protein expression by confocal laser microscopy (LSM 710). CEF cells were infected with aGM-prM + E, aGM, or JM (DTMUV) at an MOI of 0.1. The infected cells were fixed at 24 hpi, probed with anti-NDV rabbit polyclonal antibodies and anti-DTMUV mouse polyclonal antibodies, and then incubated with a DyLight 549 AffiniPure goat anti-rabbit IgG and DyLight 488 AffiniPure goat anti-mouse IgG. DAPI was used for nuclear staining.

were monitored daily. As expected, inoculation with aGM/prM + E had no adverse effects on the experimental ducks, confirming that the chimeric virus, as well as the NDV vector, were safe. In terms of the efficiency of the aGM/prM + E virus, its mean HI titer was two-fold higher than that of the LaSota vaccine at 14 dpi, suggesting that a stronger immune response could be elicited by the predominant NDV-matched vaccine, as has been reported in the literature (Hu et al., 2009; Sun et al., 2017; Xiao et al., 2012). After virus challenge, there was no viral shedding for the immunized ducks, which indicates that a two-dose immunization can yield full protection. Although the mean HI titer of the LaSota vaccine was higher than 5.0 $\log_2$, viral shedding was not prevented even though the virus was not completely shed after NDV infection (Kang et al., 2016). The sporadic reports of virulent NDV in waterfowl and outbreaks in intensive raising areas may be attributed to the lack of immunization or the efficiency of commercial vaccines. Therefore, our results provide an ideal choice for the control of ND in waterfowl. The mean VN titers of aGM/prM + E virus and HB vaccine were both less than 2.0 $\log_2$ after primary immunization, indicating

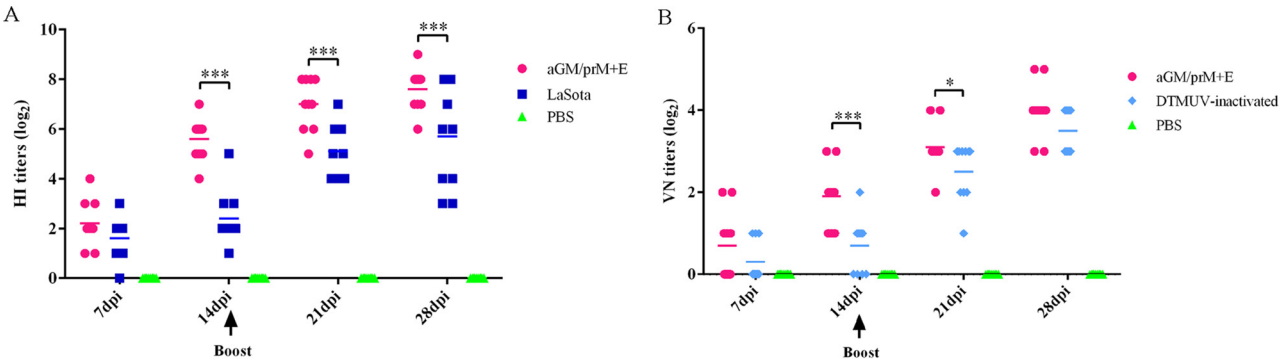

Fig. 6. Measurement of HI titers of NDV (A) and VN titers of DTMUV (B) in 150-day-old layer ducks. Groups of 10 ducks were inoculated subcutaneously with $10^{6.0}$ EID_{50} of aGM/prM + E or LaSota or PBS as a negative control. At 14 days post immunization (dpi), the ducks received a second boosting dose. The serum samples were collected every 7 days for analysis. (A) The HI titers that aGM/prM + E elicited (indicated by purple dots) were remarkably higher (***, $p < 0.001$) than LaSota (indicated by blue squares) after 14 dpi by two way ANOVA multiple comparisons statistical analysis. The results from the PBS-inoculated group were negative (less than 1.0 log_2, indicated by green triangles). The mean HI titers are highlighted with hyphen. (B) By two way ANOVA multiple comparisons statistical analysis, the VN titers that aGM/prM + E elicited (purple dots) were remarkably higher (***, $p < 0.001$) than those from the DTMUV inactivated vaccine (indicated by light blue diamonds) at 14 dpi, and significantly higher (*, $p < 0.05$) than those from the DTMUV inactivated vaccine at 21 dpi after boosting. The results from the PBS-inoculated group were negative (green triangles). The mean VN titers are highlighted with hyphen (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Viral shedding and ovarian lesions post challenge.

Vaccine group	HI titers (log_2)[a]	VN titers (log_2)[b]	Virus challenge	Viral shedding						ovarian lesions
				1 dpc		3 dpc		5 dpc		
				O[c]	C[d]	O	C	O	C	
aGM/prM + E	7.6 ± 0.8	NA[e]	NDV	0/10	0/10	0/10	0/10	0/10	0/10	NA
aGM/prM + E	NA	4.0 ± 0.6	DTMUV	0/10	0/10	1/10	4/10	NA	NA	2/10
LaSota	5.7 ± 2.0	NA	NDV	0/10	0/10	2/10	0/10	0/10	0/10	NA
HB	NA	3.5 ± 0.5	DTMUV	0/10	2/10	3/10	6/10	NA	NA	4/10
PBS	< 1	NA	NDV	1/10	0/10	5/10	2/10	0/10	0/10	NA
PBS	NA	< 1	DTMUV	0/10	3/10	3/10	8/10	NA	NA	10/10

[a] HI titers indicates mean ± standard deviation HI titers of NDV at 28 dpi.
[b] VN titers indicates mean ± standard deviation VN titers of DTMUV at 28 dpi.
[c] O, oropharyngeal swabs.
[d] C, cloacal swabs.
[e] NA, not applicable.

that one round of immunization was inadequate. The inactivated vaccines of most flavivirus cannot provide sufficient protection in one dose (Hu et al., 2017; Wang et al., 2015a), and many recombinant NDV-vectored vaccines require a prime-boost strategy (Duan et al., 2015). As our results showed, boost immunization of aGM/prM + E elicited a strong antibody response, resulting in VN titers against DTMUV higher than the commercially available inactivated DTMUV vaccine, and the former afforded 80% protection for ovarian lesions in layer ducks compared to 60% for the latter. As to viral shedding, the former afforded 60% protection compared to 40% for the latter. In general, the magnitude of the immune response to live viral vaccines for primary immunization is substantially greater and broader compared to subunit protein or inactivated virus vaccines (Belshe et al., 2007). Our results revealed that the prime-boost strategy has a better immune effect in terms of not only on shortening the time required to mount an effective antibody response but also strengthening its protective effects.

In conclusion, here we have developed a recombinant vaccine candidate aGM/prM + E that provides better protection than the LaSota vaccines in addition to conferring protection against the newly emerged DTMUV. The aGM/prM + E vaccine elicits a faster antibody response and reduces NDV and DTMUV shedding. A live ND vaccine has many advantages, including its ease of production and convenience of administration. Therefore, the bivalent vaccine candidate aGM/prM + E would facilitate a decrease in the transmission of NDV and DTMUV in waterfowl raising areas in China.

Conflict of interest

The authors declare no conflict of interest.

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