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Development of an attenuated live vaccine candidate of duck Tembusu virus strain

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ABSTRACT

Infection with duck Tembusu virus (DTMUV) can cause large economic losses to the duck-rearing industry in China. In this study, we isolated a virulent strain of DTMUV (SDS) from sparrows near a duck farm and attenuated it via serially passaging (alternately for 100 passages) in specific pathogen-free chicken and duck embryos. We attenuated the virus after the 60th passage (SDS-60), based on the production of embryos that were free of visible lesions and still alive. The 70th adapted strain (SDS-70), obtained with a virus titer of $10^{-2.46}$ EID_{50} was chosen to be the live attenuated vaccine. After immunizing ducklings with the SDS-70 strain, they obtained 100% protection against infection by the SDS-10 virulent strain. Our data demonstrate that the vaccine can protect ducks from becoming infected with TMUV. Our study also shows that this newly developed attenuated vaccine candidate provides excellent immunogenicity, is safe, and has the potential to control DTMUV infections in ducks.

1. Introduction

In 2010, a novel disease broke out in many duck farms across China. The disease was characterized by numerous symptoms, including a decline in egg-drop production, hyperemia, hemorrhaging, degeneration, distortion and lymphocyte infiltration into the ovaries (Su et al., 2011). The disease, diagnosed as duck hemorrhagic ovaritis, was proven to be caused by the Tembusu virus (TMUV) (Wang et al., 2011a, 2011b; Yan et al., 2011; Cao et al., 2011b), which is a mosquito-borne flavivirus, similar to other flaviviruses, such as Japanese encephalitis virus, Dengue virus, and West Nile virus (Long et al., 2007; Funk and Khromykh, 2009; Watanaveeradej et al., 2011; George et al., 2015). Duck TMUV (DTMUV) belongs to the genus *Flavivirus*, family Flaviviridae. Its DNA possesses one long open reading frame, which includes four 5′ structural genes that generate nucleocapsid, pre-membrane, membrane (M), and envelope (E) proteins and seven 3′ non-structural genes that generate NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins (Mukhopadhyay et al., 2005; Li et al., 2012; Tang et al., 2012, 2015). Among these proteins, the E protein plays a part in receptor binding, entry, and fusion in viruses. Although the TMUV was first isolated from ducks, subsequent studies found that it could be isolated from chickens, geese, penguins, and sparrows (Tang et al., 2013a). In addition to the rapid spread of the virus among duck populations, DTMUV might have the potential to infect humans, highlighting the urgent need to prevent and control the disease (Vaidya et al., 2012; Tang et al., 2013b) before it escapes from its current hosts.

Effective control mechanisms for preventing the transmission of DTMUV are urgently needed, among which the development of an effective vaccine would be particularly valuable. Effective vaccines for flaviviruses have been developed and are widely used for mammals, including vaccines developed against yellow fever and Japanese encephalitis (Nitayaphan et al., 1990; Guirakhoo et al., 2004). Adaptation and attenuation of DTMUV following serial passage in chicken embryos has been reported (Sun et al., 2014a,b); however, a vaccine candidate against DTMUV using chicken embryos and then duck embryos has not been developed.

In this study, we attenuate the SDS strain by serially passaging (alternately) specific pathogen-free (SPF) chicken embryos and duck embryos for 100 passages. The primary objectives of our study were to attenuate the virus following serial passages and changes in the nucleotide and amino acid sequences of the virus to evaluate the potential of the attenuated virus as a vaccine candidate. We finally chose the SDS-70 virus strain as the attenuated vaccine candidate, which demonstrated the efficacy of the live attenuated DTMUV. The success of

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this study provides the foundations for the prevention and control of the disease, which should be of great significance to the duck industry.

2. Materials and methods

2.1. Virus and animals

We preserved the virus we collected at the Research Institute of Poultry Disease of Shandong Agricultural University. We named the strain SDS, the gene sequence of which has been deposited with Genbank (accession number KM066945). We purchased the 9-day-old SPF chicken embryos we used in the study from the Poultry Research Institute of Shandong Academy of Agricultural Sciences, whereas we purchased the 9-day-old SPF duck embryos used in the study from Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Science in Heilongjiang Province, China. The 1-day-old ducklings used in the study were purchased from a hatchery in Taian, Shandong. All animals and embryonated eggs in the study were initially free of the specific pathogen we studied. We housed the experimental ducklings in SPF animal isolators that were ventilated under negative pressure. All subject animals were provided with feed and water *ad libitum*.

2.2. Adaptation and serial passages of SDS strain in embryonated eggs

The SDS strain we studied was serially passaged (n = 100) by alternately inoculating the strain into the allantoic cavity of 9-day-old chicken and duck embryos. The inoculated eggs were kept in an incubator for 72 h at 37 ℃ and candled daily. We recorded all lesions (hemorrhages, congestion or stunting, and death) of embryos. Allantoic fluids were collected and detected for the presence of the TMUV virus with semi-nested RT-PCR in the lab). We filtered virus-positive allantoic fluids through 0.2 μm membrane filters after each passage and tested the purity of the virus every three generations.

2.3. Virus titration of different passages

We titrated SDS-10/20/30/40/50/60/70/80/90/100 strains on 9-day-old SPF duck embryos, and viruses in each passage were subjected to a series of 10-fold dilutions from 10^{-1} to 10^{-4}. We determined egg lethal dose 50 (ELD50) for SDS-10 through SDS-50 strains and the egg infectious dose 50 (EID50) of the SDS-60 through SDS-100 strains following the method of Reed and Muench (1938). We used semi-nested RT-PCR to detect viral infection of embryonated eggs.

2.4. Immune effect evaluation of different attenuated strains

One-day-old ducklings (n = 50) were equally divided into five groups (A, B, C, D, and E). Ducklings in groups A, B, C, and D were infected by intramuscular injection with 0.5 mL of the SDS-70 strain, SDS-80 strain, SDS-90 strain, and SDS-100 strain, respectively, whereas ducklings in group E (control group) were injected with 0.5 mL of phosphate-buffered saline (PBS) solution. After immunization, we collected blood samples every 3 days from all groups. We infected ducklings in all groups (via intramuscular injection) with 1 mL SDS-10 strain on day 30 post-inoculation and monitored them daily throughout the experiment, recording morbidity and mortality.

2.5. Preparation of attenuated vaccine candidate

We collected the allantoic fluids of SDS-70 strain by centrifuging the fluid at 12,000 rpm for 10 min at 4 ℃ and then passing the supernatant through a 0.2 μm filter. We soaked the dialysis bag, which contained allantoic fluids of the SDS-70 strain, in PEG2000 to cover 3 h at 4 ℃. The allantoic fluids of SDS-70 strain were harvested under sterile conditions from the dialysis bag and stored at −20 ℃.

2.6. Purity of SDS-70 attenuated vaccine candidate

We conducted tests for microbial contaminants (*sensu* Niu et al., 2017) and conventional virus RT-PCR to confirm the purity of the SDS-70 strain. In addition, we performed a hemagglutination test on the SDS-70 strain to test for hemagglutination viruses, such as influenza virus and Newcastle disease virus.

2.7. Efficacy of SDS-70 vaccine candidate against challenged infection with the SDS-10 virus strain

2.7.1. Immunization and challenging procedures

We randomly divided 7-day-old ducklings (n = 160) into three groups: immunization group (90 ducklings), cohabitating infection group (10 ducklings), and control group (60 ducklings). The 7-day-old ducklings in the immunization group were infected with the SDS-70 virus strain via intramuscular injection at a volume of 0.3 mL. A second immunization was carried out after 14 days, whereas the control group was injected only with PBS. After being immunized, we collected blood samples and cloacal swabs from the three groups every 3 days.

Twenty ducklings in the immunization group and 10 ducklings in the control group were randomly separated and challenged intramuscularly with 1 mL of the SDS-10 strain at 7, 14, and 21 days after being vaccinated. We recorded any abnormalities (clinical signs, morbidity, or mortality) on a daily basis. Three ducklings from both the immunization group and control group were then euthanized and necropsied every 5 days after immunization. We collected tissue samples of spleen, kidney, and heart; fixed the tissues in 10% formaldehyde solution; and embedded them in paraffin. All the samples were tested separately. A 4 μm section of tissue was removed from each paraffin block and stained with hematoxylin and eosin (HE) for histopathologic examination.

2.7.2. Antibody titer

We obtained serum from all blood samples. The antibody titer was detected via the ELISA assay (Chen et al., 2014).

2.7.3. Viral load detection

We quantified viral shedding of cloacal swabs via real-time PCR (Yun et al., 2012a,b; Liu et al., 2013). Every sample was set up triplicated and a control sample.

2.8. Genomic sequencing

We extracted total viral RNA from the allantoic fluids of SDS-20/40/60/80/100 with a MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China). A conventional RT-PCR assay was performed to determine the complete genome sequence of the Tambusu virus with gene-specific primers (Table 1) (Sun et al., 2014a,b). We purified the RT-PCR products, cloned them into a pMD18-T vector (TaKaRa, Dalian, China), and sequenced them with BGI (Shenzhen, PR China).

Phylogenetic analyses of SDS-20/40/60/80/100 and SDS sequences were conducted with the MEGA version 7.0 program using the neighbor-joining method and the bootstrap validation method with 1000 replications.

3. Results

3.1. Attenuated by serial passage

Inoculated embryos with SDS-10 hemorrhaged and died within 72–90 h after incubation. By the 50th-passage, we observed slight hemorrhaging of body parts, brain, and liver; death occurred within 90˜120 h post-infection. The ELD50 of SDS-10 through SDS-50 strains ranged from 2.9 to 2.6 $\log_{10}ELD_{50}$/0.2 mL (Table 2). After the 60th-passage, embryos were free of visible lesions and living; the EID50 then

Table 1
Primers used for the complete genome amplification of SDS.

Fragments	Primers	Sequences(5'to 3')	Product size
F1	33F	GCTTTTGGAGTAGTGCG	1185bp
	1217R	CATAAGTTGCCTTGGGAT	
F2	1279F	TTTGTTCGGAAAGGGRAGYATA	1002bp
	2280R	CTTTKAGTGTTGACGTGAARGC	
F3	2004F	CACCAGTTGGACGCTTGATA	2013bp
	4016R	GCATGCCTCCCTTCTTTTTT	
F4	3938F	GTGTTTGAAGGGACGGTTAG	1657bp
	5594R	TGTCTGTTATTGGCGAGTTG	
F5	5568F	CGGACTCCAACTCGCCAAT	743bp
	6310R	AGAACAGTATTGCATGAGGG	
F6	6271F	GCGGTGGTGCTTTGATGG	1998bp
	8268R	CACCTCCCATTTCAGCTGC	
F7	8070F	CATCTGACACACTGCTGT	946bp
	9016R	GTAAATGCAGGTCTCACA	
F8	8993F	GGGAAGTGTGAGACCTGC	1974bp
	10967R	GCCACACTTTCGGCGATC	

ranged from 2.9 to 2.3 $\log_{10}EID_{50}$/0.2 mL (Table 2).

3.2. Immune effects of various attenuated strains

In order to identify the best vaccine candidate, we performed immune efficacy evaluations for SDS-70/80/90/100. After being challenged by the virus, one to two ducklings of groups B, C, and D and seven ducklings of control group E exhibited neurological symptoms. However, ducklings of group A remained healthy. Therefore, the SDS-70 strain was prepared as a candidate for the live attenuated DTMUV vaccine.

3.3. Purity of SDS-70 vaccine candidate

We determined that the SDS-70 strain had no microbial contaminants (e.g., bacteria and mold growth) and that it was free of other pathogens.

3.4. Efficacy of SDS-70 vaccine candidate against infection with SDS-10 strain

3.4.1. Histopathological lesions of ducklings immunized with the SDS-70 strain

All ducklings remained healthy throughout the experiment without showing any clinical signs of disease. Upon being necropsied, spleens showed slight swelling, whereas the swelling of the spleen became subdued and even vanished as the length of time since immunization increased (Fig. 1). Slight hemorrhaging occurred in the spleen and kidney, but all lesions faded and vanished as the length of time since immunization increased (Fig. 2A–H and Fig. 2a–h). Slight swelling occurred on cardiac muscles throughout the experimental period (Fig. 2I–P).

3.4.2. Challenge protection

To investigate the protective efficacy of the vaccine candidate developed in the study, we challenged ducklings with the virus at different points in time after being vaccinated (Table 3). In the immunization group, ducklings immunized with the SDS-70 vaccine obtained a protection of 100% with no morbidity, mortality, or any gross or histopathological lesions. However, ducklings in the unvaccinated control group obtained minimum protection, with 30% of ducklings developing cerebral hemorrhages, splenomegaly, extreme morbidity, and mortality.

3.5. Antibody response

The induced antibody response in the immunization group rose rapidly from 0.07 to 1.13 μg/mL by 1 day post-inoculation (dpi) and peaked at 1.55 μg/mL by 16 dpi. The induced antibody response in the immunization group was significantly higher relative to the cohabitation group throughout the period of study. Furthermore, the induced antibody response in the control group did not rise significantly throughout the period of study. The antibody level of three groups differed significantly ($P < 0.01$) (Fig. 3A).

3.6. Viral load detection

We detected viral shedding of cloacal swabs at 1 dpi, in the immunization group, which was maintained at about 80%. We detected viral shedding of cloacal swabs of the cohabitation infection group from 4 to 34 dpi, which rose from 65.9% to 98%. The relative viral loads of the cloacal swabs indicated that ducklings shed the virus for a long time, at least as long as our experiment lasted (Fig. 3B).

3.7. Alignment and phylogenetic tree

By aligning the nucleotide sequences of complete genome, we generated phylogenetic trees and confirmed the evolutionary relationships between the SDS strain and other attenuated virus strains (Fig. 4). We found that the SDS-20 strain was closely related to the SDS strain and that the two strains were in the same clade, whereas the SDS-40/60/80/100 strains shared a distant relationship with the SDS strain.

3.8. Genome sequence analysis

Substitutions of 33 bases occurred in SDS-20/40/60/80/100 strain sequence (relative to the SDS strain sequence), which included 15 neutral mutations and 18 amino acid mutations. Nucleotide base substitution at position 338 (C–T) within the C protein was identified in SDS to the SDS-20 strain following passages. Additional two nucleotide bases were substituted at position 373 (C–G) within the C protein and at position 10,956 (G-C) within 3′-UTR in the viral genome. We identified a nucleotide base substitution at position 373 (G-C) within the C protein in the SDS-20 to SDS-40 strains. Additional four nucleotide bases were substituted in the viral genome: at position 1123 (G-A) within the E protein, at position 3001 (C-A) within the NS1 protein, at position 5935 (T-C) within the NS3 protein, and at position 10,701 (A–C) within the 3′-UTR. We identified nucleotide base substitutions at position 979 (C–T) and position 1693 (C–T) within the E protein and in the SDS-40 to SDS-60 strains. We identified nucleotide base substitution at position 2401 (T-A) within the E protein and at position 9730 (G-A) within the NS5 protein the SDS-60 to SDS-80 strains. We identified nucleotide base substitution at position 259 (C–T) within the C protein, at position 2257 (A–G) within the E protein, and at position 10,243 (C–T) within the NS5 protein in the SDS-80 to SDS-100 strains (Table 4).

We identified amino acid change at position 375 (V-M) within the E protein in the SDS to SDS-20 strains. Two additional amino acid changes were identified at position 306 (Y-H) within the NS4B protein and at 904 (V-L) within the NS5 protein. There were eight amino acid

Table 2
Neutral mutations in viruses of different serial passages of SDS.

generation	10	20	30	40	50		60	70	80	90	100
ELD_{50}(0.2 mL)	$10^{-2.9}$	$10^{-2.8}$	$10^{-2.8}$	$10^{-2.8}$	$10^{-2.6}$	EID_{50}(0.2 mL)	$10^{-2.9}$	$10^{-2.46}$	$10^{-2.8}$	$10^{-2.3}$	$10^{-2.56}$

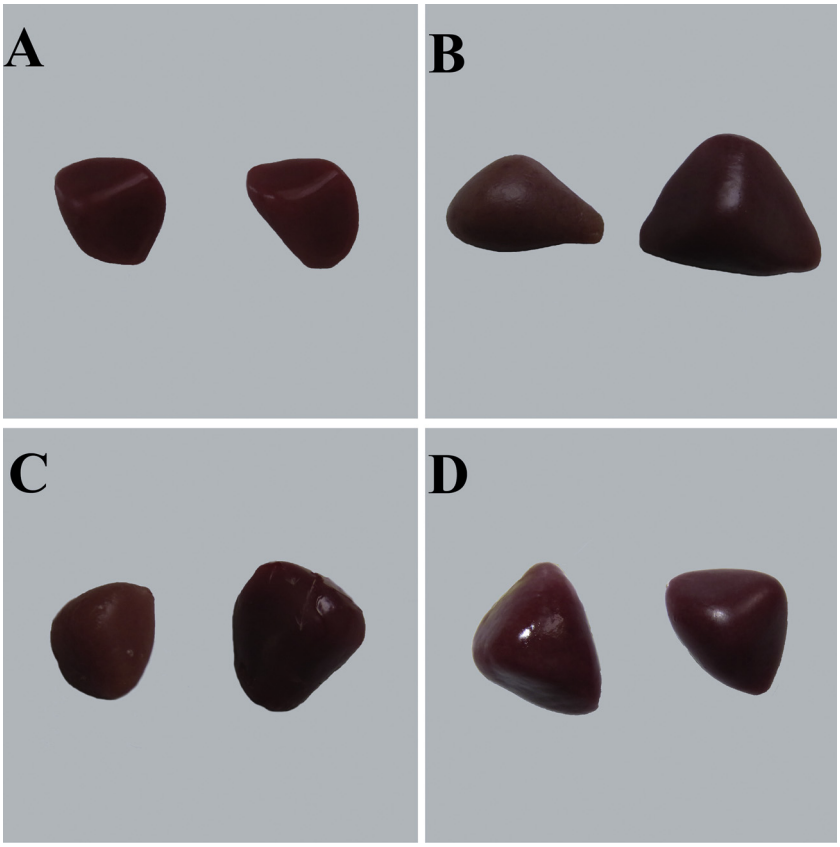

Fig. 1. Spleen changes after immunization (the left panel for the control group and the right panel for the immune group): A: 1 day post-immunization; B: 6 days post-immunization; C: 11 days post-immunization; and D: 16 days post-immunization.

changes in the SDS-20 to SDS-40 strains, which were located at position 85 (A–V), 97 V-M, and at position 341 S-F) within the E protein, at position 1 (G–R) within the NS3 protein, at 72 (V-A) within the NS4A protein, at positions 83 (K-E) and 306(H–Y) within the NS4B protein, and at position 283 (A–S) within the NS5 protein. Four amino acid changes were at positions 10 (D–G), 19 (E–G), 56 (V-I), 341 (F–S), and 441 (G-D) within the E protein in strains SDS-40 to SDS-60. An amino acid change at position 154 (N-H) within the E protein was identified in strains SDS-60 to SDS-80. Additional three amino acid changes were located at position 146 (T-S) within the NS2A protein, position 85 (M-L) within the NS4A protein, and position 898 (E–G) within the NS5 protein (Table 5).

A deletion of an N-linked potential glycosylation site appeared on the amino acid sequence of protein E in the SDS-80 strain, which we predicted using the NetNGlyc 1.0 Server (Fig. 5). According to our analysis of amino acid changes, the amino acid substitution was at position 154 (N-H) within protein E in the SDS-80 strain, which triggered the deletion of the N-linked potential glycosylation site. The amino acid substitution was at position 441 (G-D) within the E protein in the SDS-60 strain, which caused an antigenic index mutation on the gene *E* of the SDS-60 strain (Fig. 6).

4. Discussion

The outbreak and quick spread of DTMUV, which is mainly caused by hemorrhaging ovaries, has brought an enormous economic loss to the duck industry of China. Therefore, the development of an effective vaccine is sorely needed. Live attenuated virus vaccines are more advantageous than inactivated vaccines; immunity can rapidly occur, and immune cells can quickly reach the respiratory tract mucosa or digestive tract mucosa, which play an important immunoprotective role. A serial passage of parental flaviviruses is often used in developing live attenuated vaccine candidates against the virus. Effective vaccines for flaviviruses have been developed and widely used for mammals, including those against the yellow fever virus and the Japanese encephalitis virus (Nitayaphan et al., 1990; Guirakhoo et al., 2004).

In this study, the SDS strain was attenuated by passaging serially in SPF chicken embryos and duck embryos (alternately) for 100 passages. After the 60th passage, embryos were free of visible lesions and alive. Our experiment evaluating the immune efficacy of SDS-70/80/90/100 showed that the SDS-70 strain had attenuated and retained immunogenicity suitable as a DTMUV vaccine candidate. We could observe hemorrhaging in the spleen in the post-inoculation of SDS-70, but the hemorrhaging eased and disappeared with the passage of time. A popular explanation is that virus can replicate in the cells of the spleen.

We developed the SDS-70 attenuated vaccine candidate and evaluated its efficacy and safety in protecting ducks from TMUV infection. Our ELISA results showed that the antibody level in immunized ducklings increased rapidly at 1 dpi. However, the antibody response produced by immunization is retained in the body for only a short time because it is quickly metabolized. Multiple immunizations are required to offset for this deficiency. After a second immunization, the level antibodies in ducklings remained high over the whole period of growth and the vaccine provided complete clinical protection against infection with the virulent SDS-10 strain.

Viral shedding of cloacal swabs of the immunization group was detected at 1 dpi, which indicates that the virus can replicate in ducks. The antibody of the cohabitation infection group maintained at a low level throughout the period of study. Therefore, it is clear that for ducklings unvaccinated in practice, cohabitation infection can offset a lack of immunization.

The TMUV viruses are primarily transmitted by mosquitos and sparrows and the virus can infect many species of animals, including ducks, geese, and chickens (Yun et al., 2012a,b; Zhu et al., 2012; Li

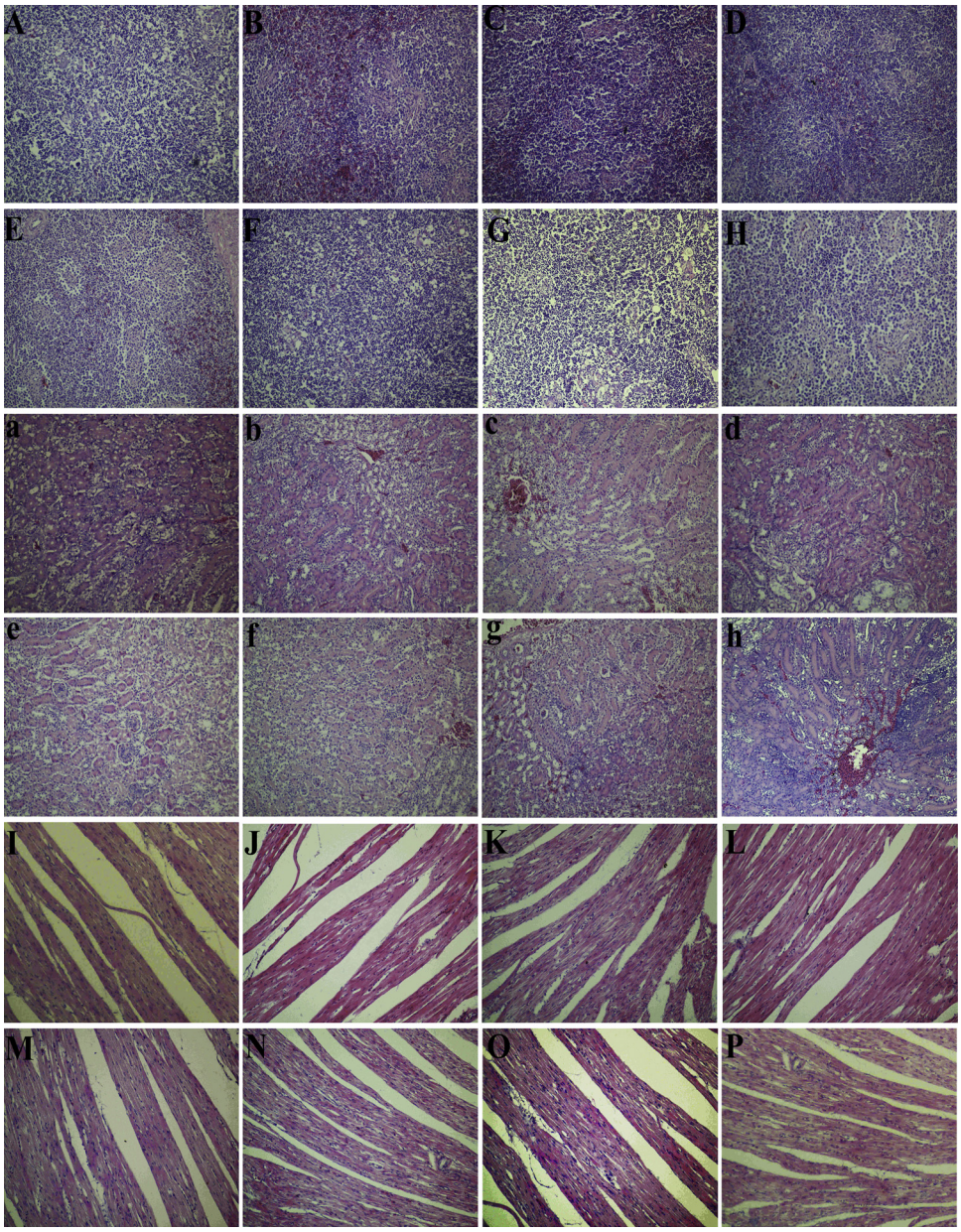

Fig. 2. HE-stained spleen section showing mild hemorrhaging: A: 1 day post-immunization; B: 6 days post-immunization; C: 11 days post-immunization; D: 16 days post-immunization; E: 21 days post-immunization; F: 26 days post-immunization; G: 31 days post-immunization; and H: 36 days post-immunization. HE-stained kidney section showed mild hemorrhages: a: 1 day post-immunization; b: 6 days post-immunization; c: 11 days post-immunization; d: 16 days post-immunization; e: 21 days post-immunization; f: 26 days post-immunization; and g: 31 days post-immunization; h: 36 days post-immunization. HE-stained cardiac muscle section showed mild swelling: I: 1 day post-immunization; J: 6 days post-immunization; K: 11 days post-immunization; L: 16 days post-immunization; M: 21 days post-immunization; N: 26 days post-immunization; O: 31 days post-immunization; and P: 36 days post-immunization.

et al., 2013). On the basis of a previous epidemiological study of a possible infection in duck industry workers in Shandong Province, China, of 132 serum samples tested, 95 (71.9%) had TMUV antibodies, and in oral swabs detection, 63 (47.7%) samples were positive for viral RNA (Tang et al., 2013b), indicating that DTMUV might pose a threat to human health. Therefore, it seems to be of great importance to preventing and control the spread of TMUV infection. In this study, ducklings immunized with the SDS-70 vaccine acquired 100% protection against TMUV. Thus, we believed that this vaccine could play a significant role in preventing the disease from spreading from fowl to humans.

The TMUV includes three structural proteins [capsid (C), membrane (PrM and M) and envelope (E)] and seven non-structural proteins (NS1, NS2, NS2B, NS3, NS4A, NS4B, and NS5). Among the structural proteins, protein E plays an important role in the host-specific adaption and attenuation of the virus, because protein E mediates the essential functions of attachment to and fusion with, host cell membranes (Ma et al., 2016; Zhou et al., 2016). In our genomic sequence analysis, the amino

Table 3
Protective efficacy of SDS-70 vaccine against SDS-10 virus strain challenge.

Group	Number of ducklings	Challenge at (dpi)	Number of attacks	virus	Protection rat	Incidence rat
Immunization group	60	7	20	SDS-10	100%	/
		14	20		100%	/
		21	20		100%	/
Control group	30	7	10		/	30%
		14	10		/	30%
		21	10		/	30%

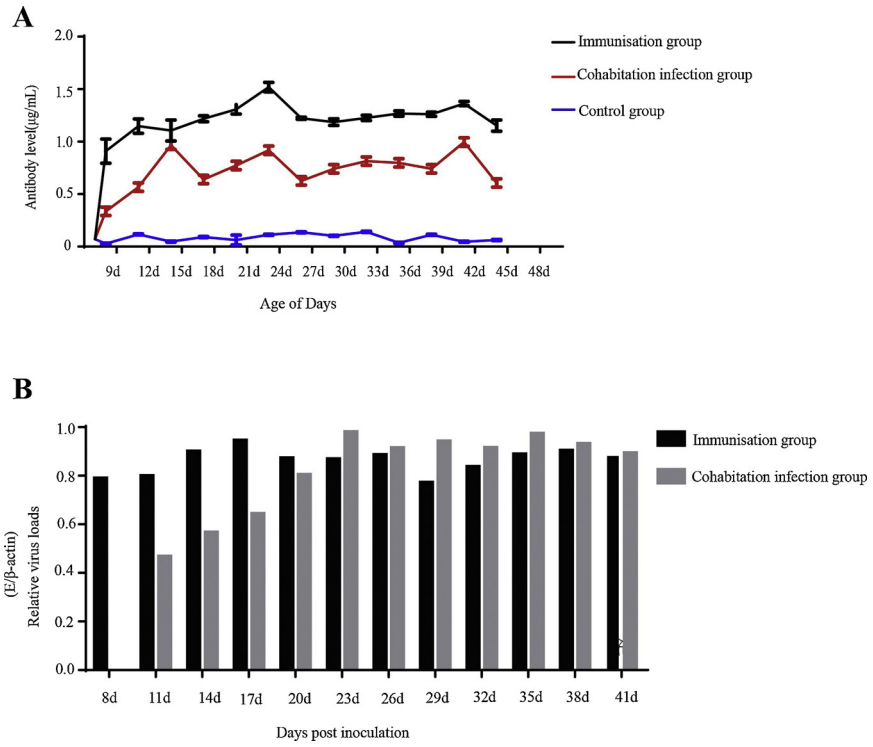

Fig. 3. Dynamic changes A: in antibody levels and B: in detoxification.

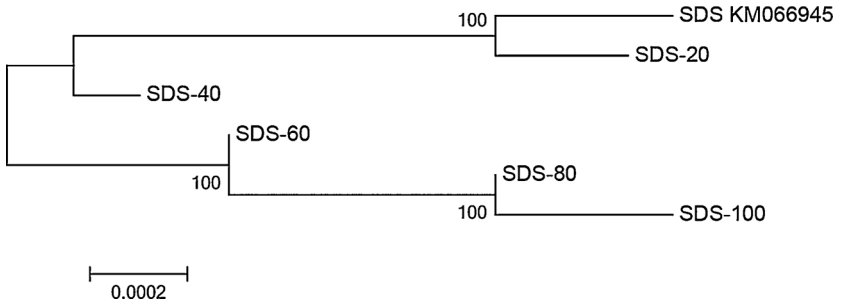

Fig. 4. Phylogenetic trees constructed using the neighbor-joining method, based on nucleotide sequences of the SDS and the attenuated SDS-20/40/60/80/100 strains.

Table 4
Neutral mutations in viruses of different serial passages of SDS.

Generation				Nucleotide substitution sites in the whole genome											
	C			E						NS1	NS3	NS5		3′-UTR	
	259	338	373	979	1123	1507	1693	2257	2401	3001	5935	9730	10243	10701	10956
SDS	C	C	C	C	G	T	C	A	T	C	T	G	C	A	G
SDS-20	C	T	G	C	G	C	C	A	T	C	T	G	C	A	C
SDS-40	C	T	C	C	A	C	C	A	T	A	C	G	C	C	C
SDS-60	C	T	C	T	A	C	T	A	T	A	C	G	C	C	C
SDS-80	C	T	C	T	A	C	T	A	A	A	C	A	C	C	C
SDS-100	T	T	C	T	A	C	T	G	A	A	C	A	T	C	C

Table 5
Amino acid mutations in viruses of different serial passages of SDS.

generation	Amino acid mutations																	
	E									NS2A	NS3	NS4A		NS4B		NS5		
	10	19	56	85	97	154	341	375	441	146	1	72	85	83	306	283	898	904
SDS	D	E	V	A	V	N	S	V	G	T	G	V	M	K	Y	A	E	V
SDS-20								M							H			L
SDS-40				V	M		F				R	A		E	Y	S		V
SDS-60	G	G	I				S		D									
SDS-80							H			S			L				G	
SDS-100																		

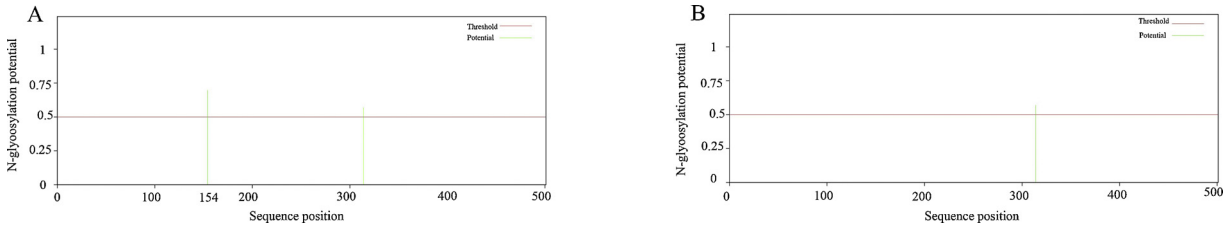

Fig. 5. Predicted *N*-glycosylation in *E* gene A: of SDS, SDS-20, SDS-40, and SDS-60 and B: of SDS-80 and SDS-100.

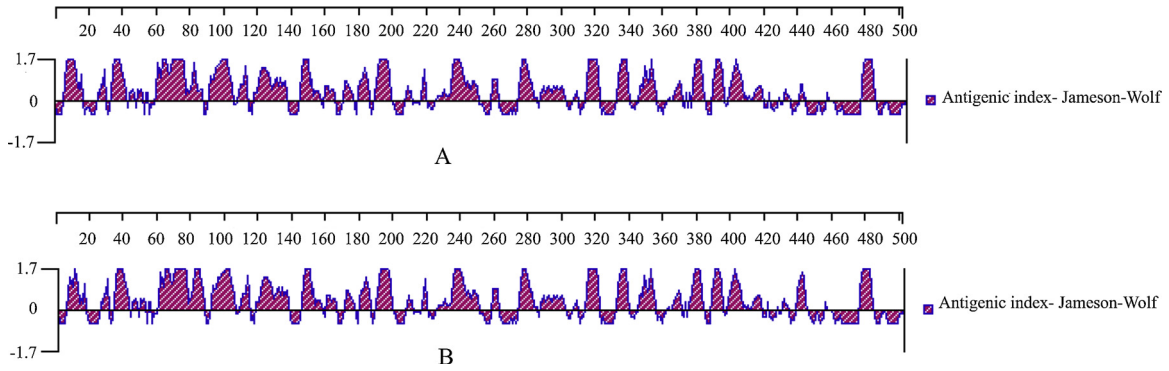

Fig. 6. Analysis of *E* gene antigen index A: of SDS, SDS-20, and SDS-40 and B: of SDS-80 and SDS-100.

acid substitution was at position 441(G-D) within protein E in the SDS-60 virus strain, which caused an antigenic index mutation. However, after the 60th passage, embryos were free of visible lesions and alive. Thus, it is possible that the antigenic index mutation plays a role in the replication and pathogenic mechanisms of DTMUV. In addition, glycosylation sites play an important role in protein structure and function (Cao et al., 2011a). Studies have shown that variation in glycosylation sites can change the virulence or antigenicity of viruses. In our sequence analysis, an amino acid change was observed at position 154 (N-H) within the protein E in the SDS-80 strain, which caused a deletion of the glycosylation site. The result suggests that the loss of the glycosylation site might be important for virulence of strain SDS.

Several other aspects need to be examined in future studies. First, although we employed intramuscular inoculation in the current study, other vaccination methods should be tested with the candidate vaccine. Second, although vaccination with the SDS-70 strain provided effective protection against the SDS-10 strain, practical investigations of the SDS-70 strain should be undertaken to determine if the attenuated vaccine will be an appropriate candidate for commercial production, such as studies on the effects of the vaccine on the production performance of egg-laying ducks. Third, in this study we did not test if the SDS-70 vaccine would interfere with other vaccines typically administered for duck diseases, but potential interference should be investigated. Nonetheless, our study has provided compelling evidence that the SDS-70 attenuated vaccine candidate could help control and prevent DTMUV infection.

Author contributions

Conceived and designed the experiments: YD YT DH. Performed the experiments: DH XZ LC. Analyzed the data: DH XZ. Contributed reagents/materials/analysis tools: DH XZ. Wrote the paper: DH.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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