Virus Research 273 (2019) 197739

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

# Attenuation of duck Tembusu virus ZJSBL01 strain following serial passage in BHK-21 cells supplied with 5-Fluorouracil

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ARTICLE INFO

*Keywords:*
Duck Tembusu virus
DTMUV
Attenuation
Vaccine

ABSTRACT

Duck Tembusu virus (DTMUV) is a new pathogen that produces an acute and potent disease in ducks which has caused serious economic losses in China. In this study, a virulent strain of DTMUV, designated as ZJSBL01, was attenuated by serial passages in BHK-21 cells supplied with 5-Fluorouracil (5-FU) for 50 passages to induce mutation and attenuation. Growth kinetics of different passages of ZJSBL01 strain in BHK-21 cells show that these viruses have similar replication characteristics. The virus was highly attenuated after 40 passages in BHK-21 cells supplied with 5-FU, based on mortality, morbidity, and viral load in inoculated Sheldrake ducklings. In addition, all of the ducklings immunized with ZJSBL01-P40, the virus obtained at passage 40 of ZJSBL01, showed seroconversion on day 14 post inoculation. Moreover, P40 did not cause clinical symptom for layding ducks. Immunization with ZJSBL01-P40 could provide effective protection against the virulent parental ZJSBL01 strain. Seventeen amino acid substitutions were observed in the polyprotein of ZJSBL01-P40 compared with parental ZJSBL01. These results indicate that ZJSBL01-P40 may be a live vaccine candidate for prevention of DTMUV-disease.

## 1. Introduction

Since April 2010, a serious infectious duck disease, characterized by egg production dropping, emerged around the main duck-producing regions of China, which has caused huge economic losses to the duck industry (Su et al., 2011; Yan et al., 2011; Yan et al., 2016). The causative agent was subsequently identified as duck Tembusu virus. In clinical practice, DTMUV infections in ducklings mainly cause viral encephalitis (Jingliang et al., 2011; Yun et al., 2012), whereas cause serious egg production dropping when infections in laying ducks (Li et al., 2013; Liu et al., 2012; Shen et al., 2016; Vaidya et al., 2012). To date, the disease has affected geese, chickens, pigeons and house sparrows (Dai et al., 2015; Li et al., 2013; Tang et al., 2013a, b; Tao et al., 2012; Vaidya et al., 2012). As a member of *Flavivirus*, DTMUV might have the potential to infect humans, indicating the potential threat to public health (Tang et al., 2013a, b).

DTMUV belongs to the family *Flaviviridae*, genus *Flavivirus*, and virus group Entaia (Pixi et al., 2011). The viral genome is a 10,990 nucleotide positive single-stranded RNA that contains an open reading frame encoding a 3425 amino acid polyprotein, which is processed into three structural proteins (C, PrM, and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Liu et al., 2013; Tang et al., 2012; Zalán Gábor et al., 2014). The envelope (E) protein of the *Flavivirus* contains virus antigenic determinants that induces the production of neutralizing antibodies (Peipei et al., 2012; Suchetana et al., 2005).

Effective control approaches should be used for prevention of DTMUV-diseases. Several live attenuated vaccines have been commercialized to protect human and livestocks against *Flaviviruses* (Benzarti et al., 2019). A live attenuated vaccine (FX2010-180 P strain) (ZHENGYE, Jilin, China), which was generated through serial passage in chicken embryo fibroblasts (Li et al., 2014), has been used for provention and control of DTMUV infection. Other attenuated strains were also developed by serial passage in chicken and duck embryos, including SDS and Du/CH/LSD/110128 strains. BHK-21 cells have been widely used in vaccine production, which suggests the necessity of development of an BHK-21 cells adaptive vaccine against DTMUV.

In 2014, a DTMUV strain, named ZJSBL01, was isolated from a commercial Muscovy duck farm in Zhejiang province of China, in which the laying ducks exhibited egg production dropping and oophoritis. 5-

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https://doi.org/10.1016/j.virusres.2019.197739
Received 1 July 2019; Received in revised form 3 September 2019; Accepted 3 September 2019
Available online 04 September 2019

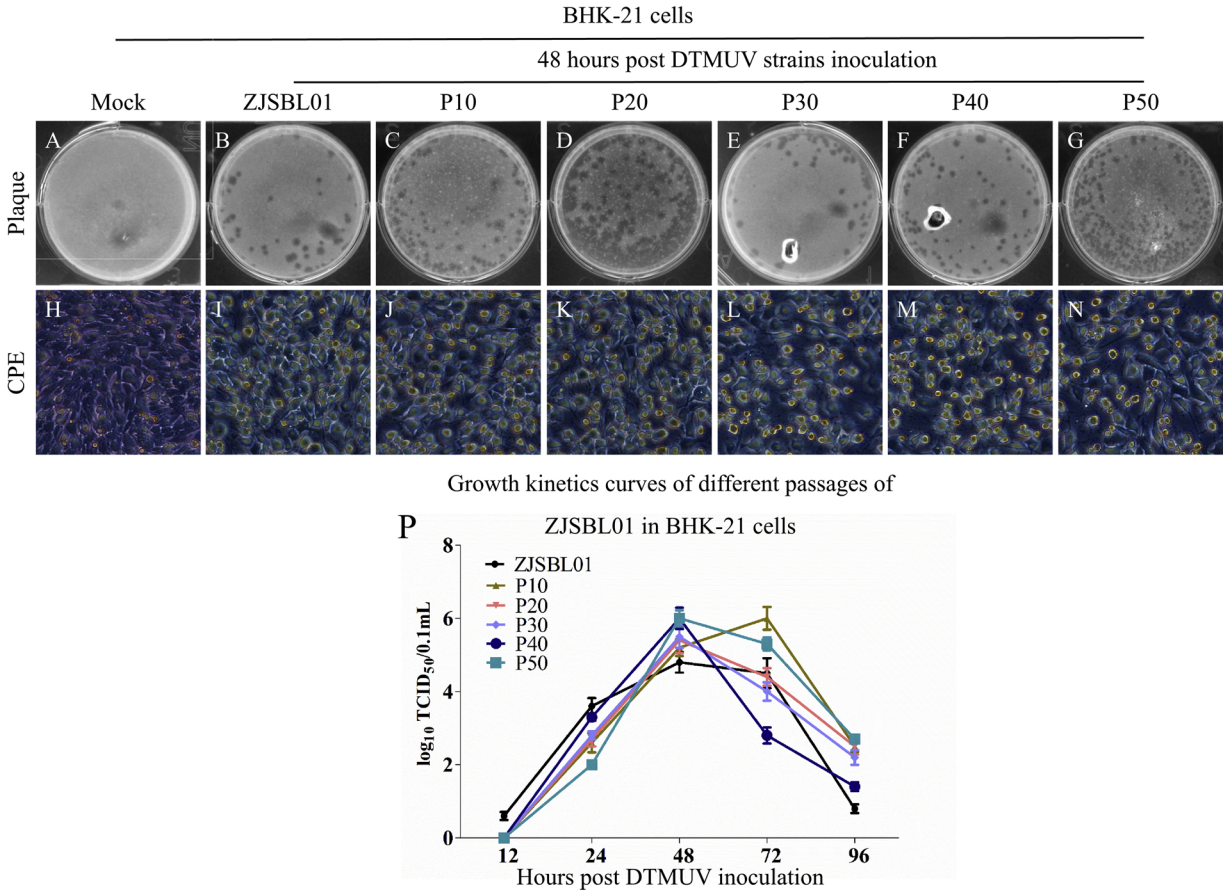


**Fig. 1.** Characterization of different passages of DTMUV ZJSBL01. (A–G) plaque purifications of different passages of ZJSBL01 in BHK-21 cells. (H–N) CPE of different passages of ZJSBL01 in BHK-21 cells. (P) Growth kinetics curves of different passages of ZJSBL01 in BHK-21 cells.

Fluorouracil (5-FU) can inhibit DNA and RNA synthesis through inhibition of thymidylate synthase (Longley et al., 2003) and it could be used as a mutagen to develop a live attenuated vaccine (Pringle et al., 1993). In this study, a DTMUV attenuated strain, ZJSBL01-P40, was developed by serial passage in BHK-21 cells supplied with 5-fluorouracil (5-FU). ZJSBL01-P40 retains the immunogenicity and protects the immunized ducklings against parental ZJSBL01 challenge. The development of BHK-21 cells adaptive ZJSBL01-P40 might be a vaccine candidate for DTMUV disease prevention.

## 2. Materials and methods

### 2.1. Virus and cells

The DTMUV ZJSBL01 strain was provided by the production management office of the Poultry Industry Division of Wen's Foodstuffs Group Co.,Ltd and stored at −80 °C until further use.

BHK-21 cells (ATCC CCL-10) obtained from American Type Culture Collection were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco) in 5% $CO_2$ incubator. The maintenance medium for DTMUV propagation was DMEM supplemented with 2% FBS.

### 2.2. Virus propagation

Monolayer cultures of BHK-21 cells were incubated with ZJSBL01 for 1 h at 37 ℃, then overlaid with maintenance medium (DMEM with 2% FBS) containing 50 μg/mL 5-FU. Cultures were incubated for 2 days at 37 °C in 5% $CO_2$ until 50% of the cells exhibited cytopathic effects (CPE). The supernatant fluid was collected and stored at −80 °C, or used immediately with 100-fold dilution for subsequent passage.

### 2.3. Plaque purification

Virus supernatants obtained at passage 10 (P10), P20, P30, P40, and P50 were serially diluted with DMEM until reaching a $10^{-4}$ dilution. Monolayers of BHK-21 cells in six-well plates were incubated with serum-free DMEM maintenance solution at 37 °C for 1 h, after which the solution was discarded. The monolayers were then inoculated with 200 μL per well of the diluted virus. Following incubation at 37 °C for 1 h, 1 mL of 2 × MEM and 1 mL 1.5% nutrient agar was added to each well, and the plates were kept flat at room temperature for 40–60 min until the agar solidified, after which they were moved to a 37 °C incubator. The morphology of the cells was observed every 12 h to assess plaque formation. Plaque-emerging agar and cells were scraped off and placed in DMEM containing 2% FBS. The collected viruses were subjected to three rounds of further purification

### 2.4. Infectious virus titrations by a $TCID_{50}$ assay

Different passages (P10, P20, P30, P40, and P50) of the ZJSBL01 were inoculated into 25-$cm^2$ culture flasks, and supernants at 12 h, 24 h, 48 h, 72 h, and 96 h post-inoculation were collected to $TCID_{50}$ determination.

BHK-21 cells were seeded in 96-well plates. When reached 60% confluence, the cells were washed three times with PBS buffer. The virus solution was diluted ($10^{-1}$–$10^{-9}$) with DMEM. Virus dilutions were then added to the cells with six replicates of 100 μL/well per dilution. Normal control cells (16 wells) were treated with 200 μL of 2% DMEM maintenance medium. After incubation in a 5% $CO_2$ incubator at 37 °C for 2 h, virus-inoculated media were removed from the wells and replaced with 2% maintenance medium. The CPE were recorded for

**Table 1**
Complete genomic analysis of the different passages of ZJSBL01.

| Gene | Mutation site | Base substitution | | | | | |
|---|---|---|---|---|---|---|---|
| | | ZJSBL01 | P10 | P20 | P30 | P40 | P50 |
| 5'U | 39 | – | – | – | – | T | T |
| C | 383 | G | G | G | G | A | A |
| | 430 | T | T | T | T | C | C |
| PrM | 458 | G | G | G | G | A | A |
| | 590 | T | T | T | T | T | C |
| | 800 | C | C | C | C | T | T |
| | 881 | C | C | C | C | T | T |
| E | 961 | G | A | A | A | A | A |
| | 1046 | T | T | T | T | T | C |
| | 1142 | G | G | G | G | G | A |
| | 1361 | C | C | C | C | C | T |
| | 1420 | A | T | T | T | T | T |
| | 1437 | C | C | C | C | C | T |
| | 1563 | G | G | A | G | G | G |
| | 1585 | G | A | G | A | A | G |
| | 1651 | C | C | C | A | A | A |
| | 1940 | T | T | T | T | T | C |
| | 2021 | G | G | G | A | A | A |
| | 2125 | A | A | A | A | G | G |
| | 2174 | A | A | A | A | G | G |
| | 2224 | A | G | G | G | G | G |
| | 2237 | T | T | T | T | T | C |
| | 2393 | A | A | A | A | A | G |
| NS1 | 2840 | G | G | G | G | A | A |
| | 2874 | A | A | A | A | G | G |
| | 2890 | C | C | T | T | T | T |
| | 2948 | G | G | G | G | A | A |
| | 2957 | G | G | A | A | A | A |
| | 2960 | A | A | A | A | A | G |
| | 3131 | A | A | A | A | G | G |
| | 3182 | C | C | C | C | T | T |
| | 3630 | T | T | T | T | T | C |
| | 3682 | A | A | A | A | G | G |
| | 3693 | C | C | C | C | T | T |
| | 3808 | A | A | A | A | A | G |
| | 4169 | C | C | C | C | C | T |
| NS2B | 4512 | C | C | T | T | T | T |
| NS3 | 4840 | A | A | A | A | G | G |
| | 4853 | A | A | G | G | G | G |
| | 5067 | G | G | G | A | A | A |
| | 5237 | A | A | A | A | G | G |
| | 5556 | A | A | A | A | A | G |
| | 5822 | C | C | C | C | T | T |
| | 5888 | G | G | G | G | A | A |
| | 6042 | G | G | A | A | A | A |
| | 6269 | C | C | T | T | T | T |
| NS4A | 6473 | C | C | C | C | T | T |
| | 6815 | G | G | A | A | A | A |
| 2K | 6833 | G | G | G | G | A | A |
| NS4B | 6995 | T | T | T | T | T | C |
| | 7175 | A | A | A | A | A | G |
| | 7571 | C | C | C | C | T | T |
| NS5 | 7718 | G | G | G | G | G | A |
| | 7952 | A | A | A | A | G | G |
| | 8291 | G | G | G | G | G | A |
| | 8354 | C | C | C | C | C | T |
| | 8392 | A | A | G | G | G | G |
| | 8414 | G | G | G | G | A | A |
| | 8484 | G | G | A | A | A | A |
| | 8494 | C | C | C | C | T | T |
| | 8529 | C | C | C | C | T | T |
| | 8699 | C | C | T | T | T | T |
| | 9614 | A | A | A | A | A | G |
| | 9707 | T | T | T | T | T | C |
| | 10143 | G | G | G | G | G | A |
| | 10156 | T | C | C | C | C | C |
| | 10226 | A | A | A | A | G | G |
| | 10310 | A | A | G | G | G | G |
| 3'U | 10374 | A | A | A | A | G | G |
| | 10437 | A | A | A | A | G | G |
| | 10448 | T | T | T | T | C | C |
| | 10606 | T | T | T | T | C | C |
| | 10647 | G | G | G | G | G | A |

different dilutions and compared with the controls. $TCID_{50}$ was calculated using Reed-Muench method.

### 2.5. One-step RT-PCR detection

Virus genomic RNA were extracted using RaPure Total RNA kits (Magen Biotech, China) according to the manufacturer's instructions. DTMUV was identified by One-step RT-PCR with the DTMUV-specific primer pair (sense: 5′-GGAGAAGAAGGAAGAAGAAGT-3′; antisense: 5′-ATGTCTGTTATTGGCGAGTT-3′). One-step RT-PCR was performed in a 25 μL volume containing 12.5 μL of 2× One-Step Buffer, 1 μL of PrimeScript One-Step Enzyme Mix, 1 μmol/L of each specific primer, and 2 μg of RNA. The thermal cycling parameters were as follows: 50 °C for 30 min, 94 °C for 5 min; 30 cycles of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 40 s, and a final extension at 72 °C for 5 min. All positive constructs were confirmed via sequencing analysis by Sangon Biotech (China).

### 2.6. Genomic analysis

Oligonucleotide primers were synthesized by Sangon Biotech according to previous study (Sun et al., 2014). The viral RNA of P10, P20, P30, P40, and P50 were extracted as above described and used for complete genome amplification by one-step RT-PCR. The PCR products were purified and cloned into pMD19-T vector (Takara, China) for sequencing analysis performed by Sangon Biotech. The complete genomic sequences were assembled and analyzed using MEGA version 7.0 program.

### 2.7. Pathogenicity analysis of different passages of ZJSBL01 in ducklings

One-day-old Sheldrake ducklings were provided by Wen's Foodstuffs Group Co.,Ltd (China). A total of 60 one-day-old ducks were randomly and equally divided into six groups. Each 10 ducklings were inoculated with $5 \times 10^5$ $TCID_{50}$ of ZJSBL01, P20, P30, P40 or P50 strain or DMEM by intramuscular injection. Clinical symptoms were monitored daily and the body weights of each duckling were determined on 7 day-post-inoculation (DPI). On 3, 5, 7, 9, and 14 DPI, Six serum samples were randomly collected from each group for the detection of neutralizing antibodies against DTMUV by competitive enzyme-linked immunosorbent assays (ELISA) as describe previously (Li et al., 2012). On 6 DPI, six serum and swab samples were randomly collected from each group for DTMUV genomic RNA copy number assessment by real-time PCR. At the same time, three birds per group were randomly selected for necropsy and clinical lesion observation in their tissues and organs, including heart, liver, spleen, lung, kidney, and brain. Corresponding tissue samples were taken for real-time RT-PCR detection, after which the spleen and brain tissue were taken for histopathological examination.

### 2.8. Pathogenicity analysis of P40 in laying ducks

To evaluate the pathogenicity of P40 in laying ducks, 120 laying Sheldrake ducks (200-day-old) with DTMUV antibody negative (Wen's Foodstuffs Group) were randomly and equally divided into three groups and raised in 3 separated negative pressure room. The ducks were inoculated with $5 \times 10^5$ $TCID_{50}$ of P40 or parental ZJSBL01 strain or inoculated with DMEM as mock inoculated group by intramuscular injection. The egg production rate was recorded daily. Three ducks from each group were randomly selected for necropsy, clinical lesion observation, and histopathological examination on 6 DPI.

### 2.9. Immunogenicity evaluation of different passages of ZJSBL01 in ducklings

A total of 60 one-day-old Sheldrake ducks were randomly and

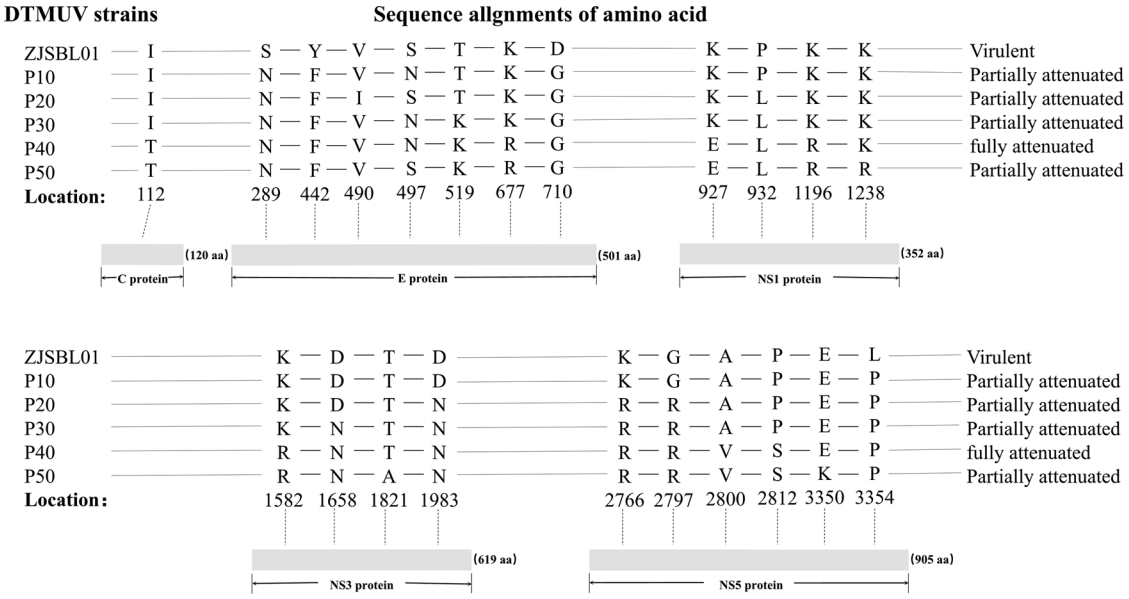


**Fig. 2.** Amino acid residues subsititution of different passages of ZJSBL01.

**Table 2**
Pathogenicity analysis of different passages of ZJSBL01 in ducklings.

| Passage | Morbidity(%) | Mortality (%) | Antibody(%) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 3[a] | 5 | 7 | 9 | 14 |
| Control | 0/10 | 0/10 | 0 | 0 | 0 | 0 | 0 |
| ZJSBL01 | 10/10 | 2/10 | 4/6 | 6/6 | 6/6 | 6/6 | 6/6 |
| P20 | 3/10 | 0/10 | 1/6 | 6/6 | 6/6 | 6/6 | 6/6 |
| P30 | 2/10 | 0/10 | 0/6 | 4/6 | 6/6 | 6/6 | 6/6 |
| P40 | 0/10 | 0/10 | 0/6 | 2/6 | 3/6 | 5/6 | 6/6 |
| P50 | 2/10 | 0/10 | 0/6 | 3/6 | 5/6 | 6/6 | 6/6 |

[a] Day post inoculation.

equally divided into six groups. Each 10 ducks were immunized with $5 \times 10^4$ $TCID_{50}$ of the P10, P20, P30, P40 or P50 strain or DMEM by intramuscular injection. Two weeks after immunization, each duck was challenged with $5 \times 10^5$ $TCID_{50}$ of ZJSBL01 strain by intramuscular injection. After administration of the DTMUV challenge, the clinical symptoms were monitored daily. On 3 and 6 day-post-challenge (DPC), six swab samples were collected for viral loads analyzed by real-time PCR. And three randomly selected birds were necropsied to monitor the changes in the tissues and organs, including heart, liver, spleen, lung, kidney, and brain. Corresponding tissue samples were taken for real-time RT-PCR detection, and the spleen and brain tissues were then taken for histopathological examination.

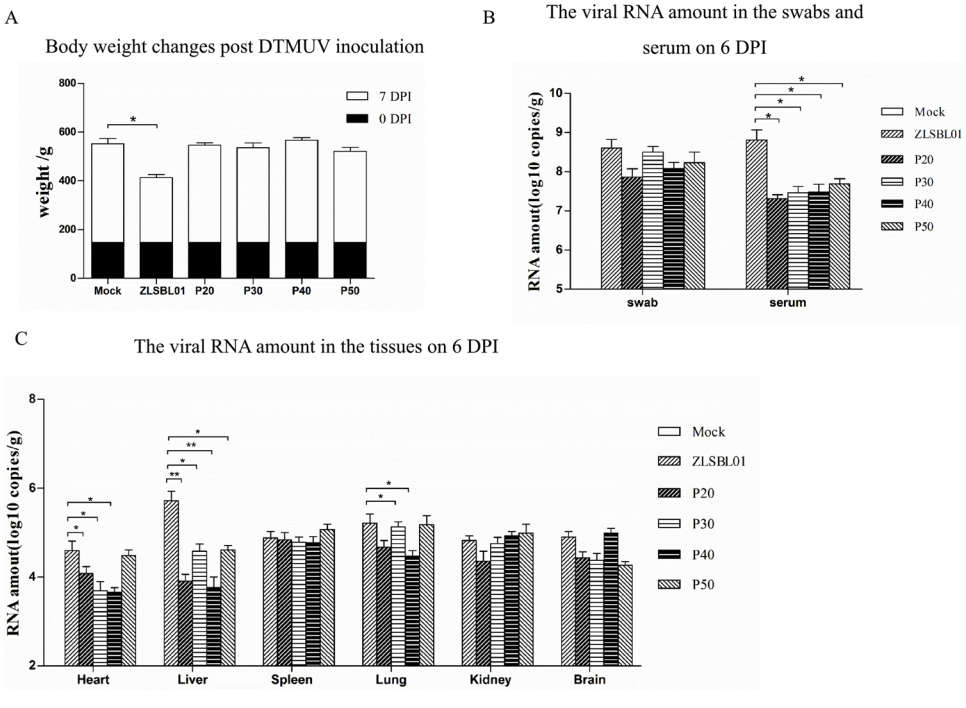


**Fig. 3.** (A) Body weight changes of the ducklings after different passages of ZJSBL01 inoculation on 7 DPI. (B) The swabs and serum viral RNA amount after different passages of ZJSBL01 inoculation on 6 DPI. (C) The viral RNA amount in the tissues after different passages of ZJSBL01 inoculation on 6 DPI. Data are represented as means ± SD. *means $p \leq 0.05$, **means $p \leq 0.01$.

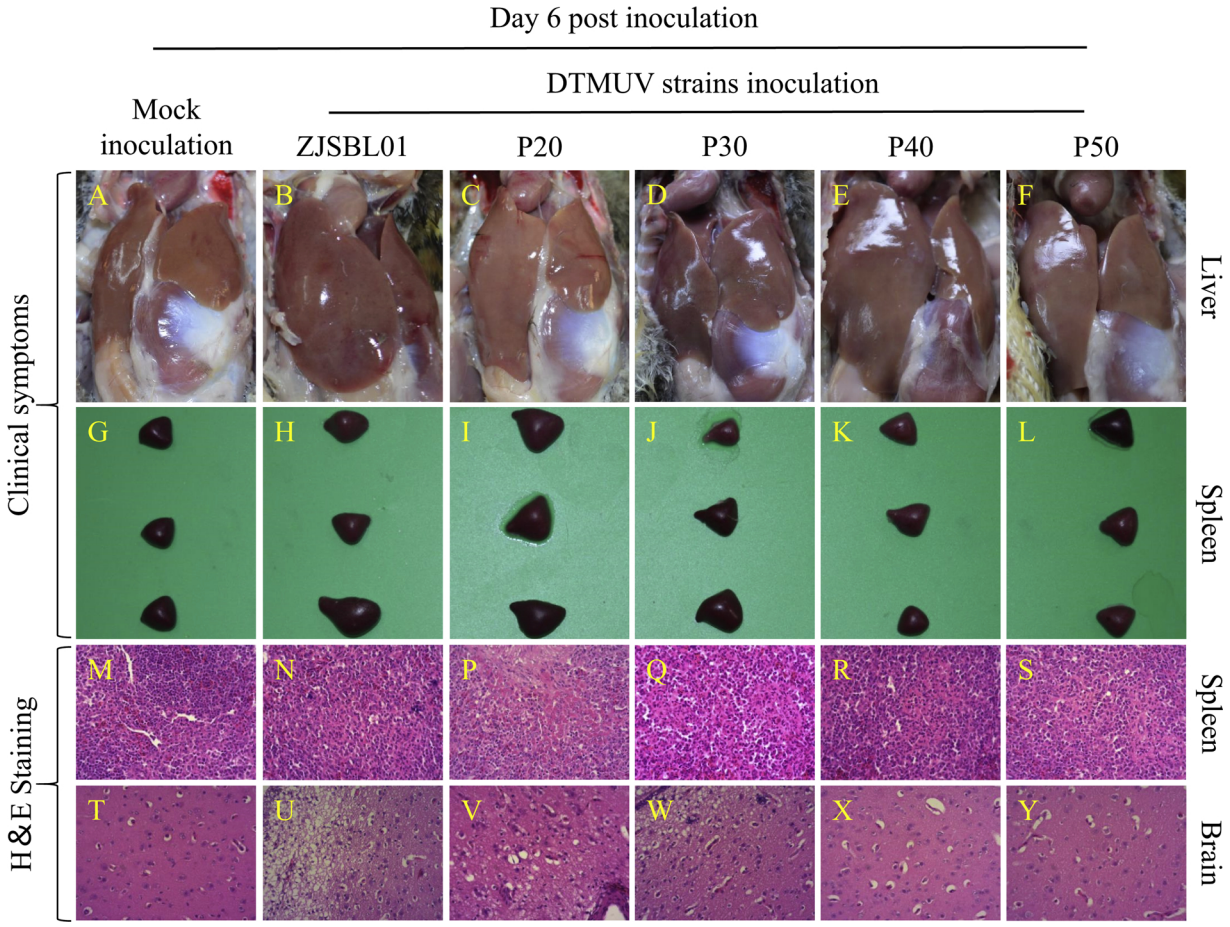


**Fig. 4.** (A–F) Clinical changes of the livers of DTMUV inoculated ducklings on 6 DPI. (G–L) Clinical changes of the spleens of DTMUV inoculated ducklings on 6 DPI. (M–R) Histological changes of the spleens of DTMUV inoculated ducklings on 6 DPI. (S–X) Histological changes of the brain of DTMUV inoculated on 6 DPI.

### 2.10. Real-time RT-PCR analysis

The serum, swab samples, and supernatants of tissues from each ducklings were centrifuged at 8000 ×*g* for 5 min. Total RNA was extracted using RaPure total RNA kits and used for reverse transcription into cDNA using PrimeScript™ RT Master Mix (Takara) according to the manufacturer's instructions. The TaqMan probe primers (sense: 5′-TGTCTTATGCAGGTACCGATG-3′; antisense: 5′-CGTATGGGTTGACTGT TATCA-3′; P: FAM-AGTTCCCATATCCATGTC-TAMRA) were synthesized by Sangon Biotech according to previous study (Yan et al., 2011). The real-time PCR assay was conducted with a 7500 Fast Real-Time PCR System (Life Technologies, America). The real-time PCR was performed in a 20 μL volume containing 10 μL of Premix Ex Taq (Probe qPCR)(2×), 0.4 μL of each specific primer (10 μM), 0.8 μL of Probe (10 μM), 0.2 μL of ROX Reference Dye II (50×)*[3], and 10 0 ng of cDNA. The thermal cycling parameters were as follows: 95 °C for 30 s; and 40 cycles of 95 °C for 5 s, 60 °C for 1 min.

### 2.11. Statistical analysis

Statistical analysis was performed utilizing the GraphPad Prism 6. The unpaired *t* test (nonparametric) was used when comparisons were made between two groups representing paired observations. The data are presented as the mean ± standard deviations (SDs). Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Plaque purification and growth kinetics in BHK-21 Cells

The ZJSBL01 strain and P10, P20, P30, P40 and P50 virus strains were plaque-purified three times, and the infected cells beneath the nutrient agar were observed under a microscope (Fig. 1A–G). All the different passages strains induced similar CPE in BHK-21 cells (Fig. 1H–N). To compare the infectivity of the different passages of ZJSBL01 virus, the $TCID_{50}$ of each generation strain was calculated by the Reed-Muench method. The results showed that the proliferation characteristics of different passages were similar to the parental ZJSBL01 strain, and each reached their maximum virus titer at 48 h post-inoculation (HPI) (Fig. 1P). The titers of the ZJSBL01, P10, P20, P30, P40 and P50 viruses when measured 48 HPI were $10^4$, $10^{4.8}$, $10^{3.7}$, $10^{5.3}$, $10^{5.7}$, $10^{5.5}$, and $10^{5.7}$ $TCID_{50}$/0.1 ml, respectively.

### 3.2. Genomic analysis

The complete genome of different passages of ZJSBL01, P10, P20, P30, P40, and P50, were sequenced and analyzed. Compared with the parental strain, 71 nucleotide substitutions (Table 1) resulting in 20 amino acid substitutions were found in P50 (Fig. 2). The structural envelope (E) protein is main immune-associated protein of DTMUV [10]. In the E protein, four amino acid residue substitutions (S289 N, Y442 F, S497 N, and D710 G) were found in P10 generation, and 4 substitutions (S289 N, Y442 F, V490I, and D710 G) in P20, 5 substitutions (S289 N, Y442 F, S497 N, T519 K, and D710 G) in P30, 6 substitutions (S289 N, Y442 F, S497 N, T519 K, K677R, and D710 G) in

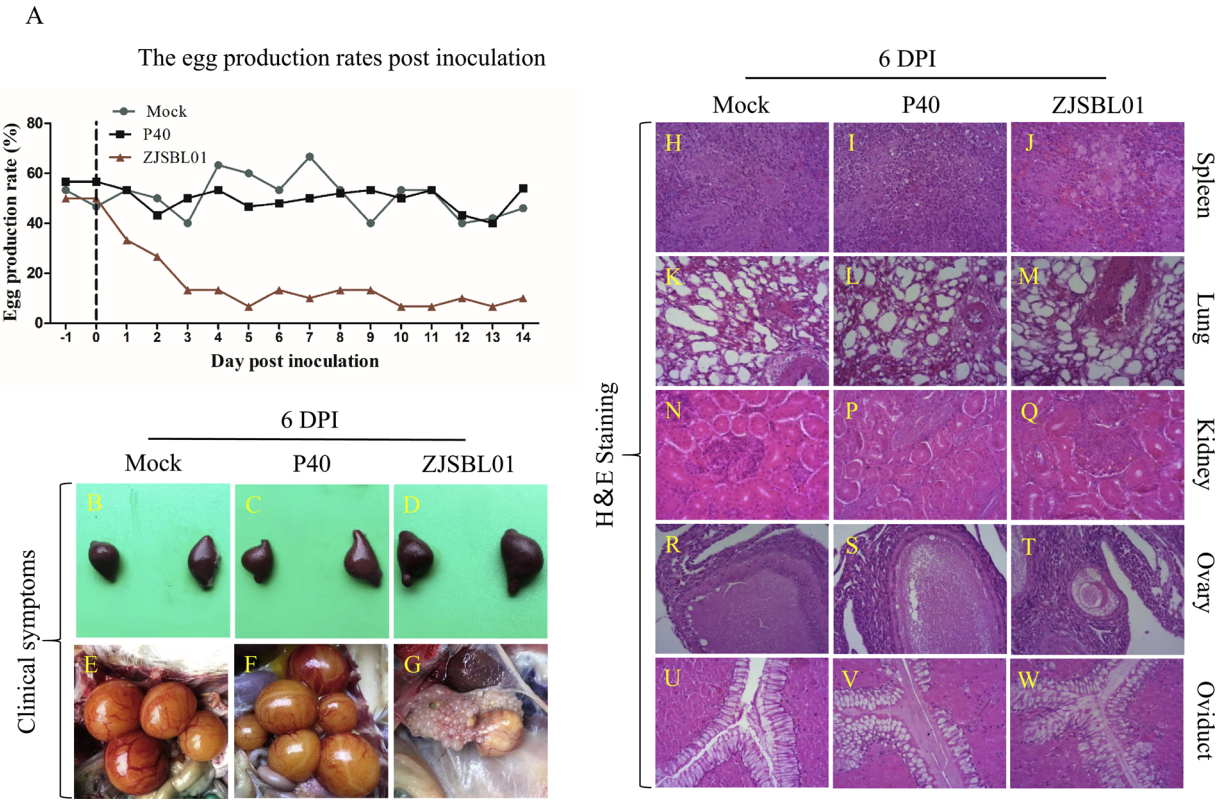


**Fig. 5.** Pathogenicity analysis of P40 strain in laying ducks. (A) The egg production rates post P40, parental ZJSBL01, or mock inoculation. (B–G) Clinical changes of the spleens and ovary at 6 days after P40, parental ZJSBL01, or mock inoculation. (H–W) Histological Changes of spleens, lungs, kidneys, ovaries, and oviducts of the P40, ZJSBL01, or mock inoculation laying ducks.

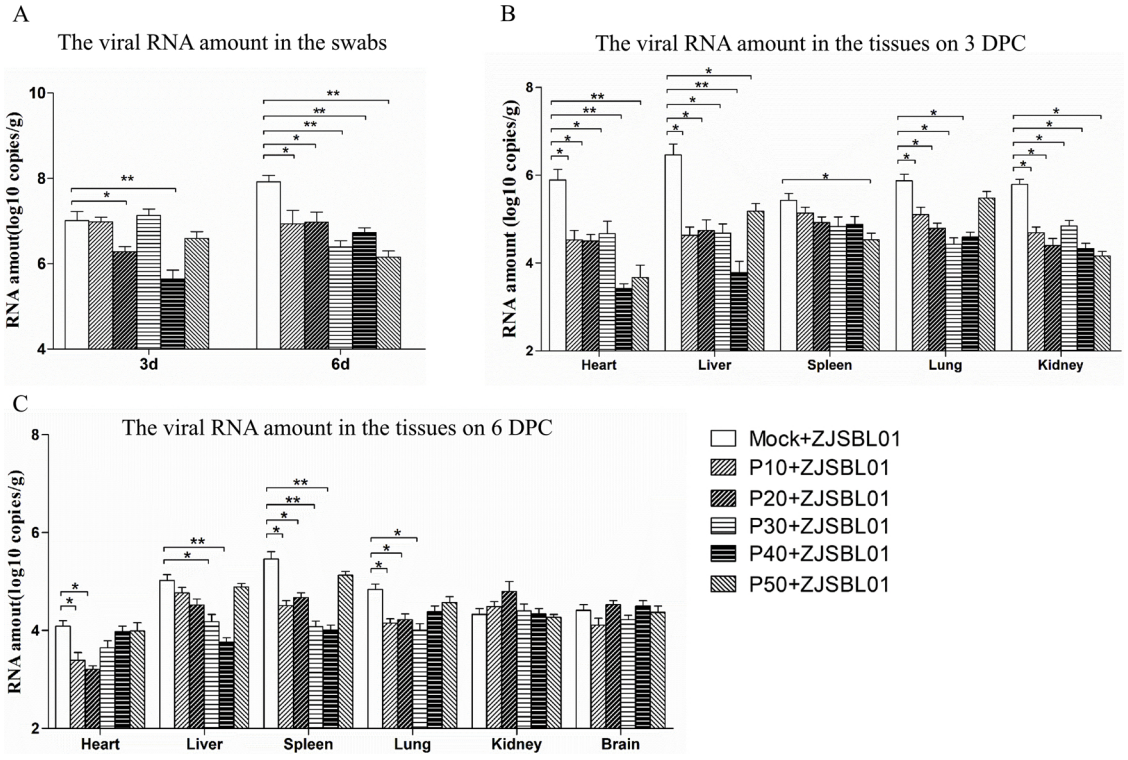


**Fig. 6.** (A) The viral loads in the swabs on 3 and 6 DPC, respectively. (B) The viral loads in different tissues on 3 DPC. (C) The viral loads in different tissues on 6 DPC. Data are represented as means ± SD. *means $p \leq 0.05$, **means $p \leq 0.01$.

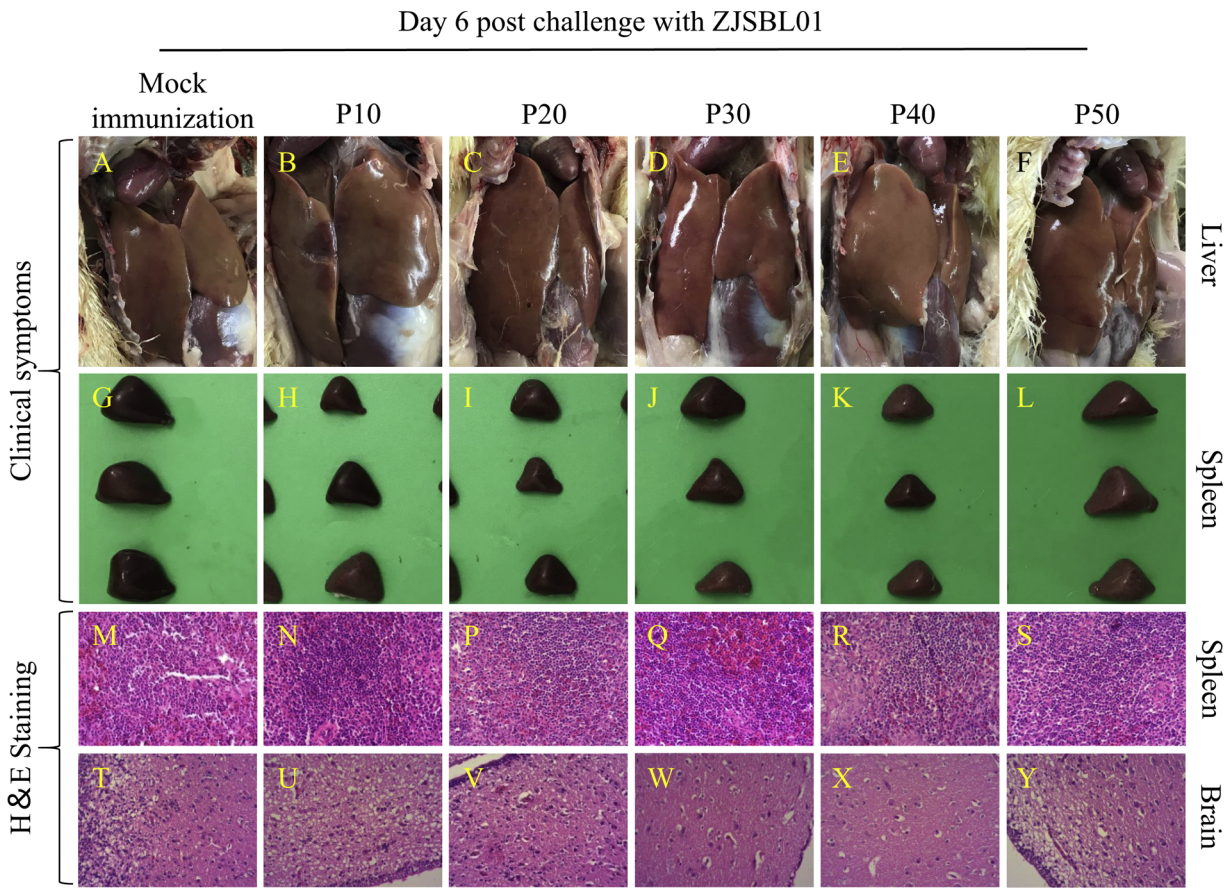


**Fig. 7.** (A–F) Clinical changes of the livers on 6 DPC. (G–L) Clinical changes of the spleens on 6 DPC. (M–R) Histological changes of the spleens on 6 DPC. (S–X) Histological changes of the brains on 6 DPC.

P40, and 5 substitutions (S289 N, Y442 F, T519 K, K677R, and D710 G) in P50 generation were also found (Fig. 2).

### 3.3. Pathogenicity analysis of different passages of ZJSBL01 in ducklings

All of the ducklings in the parental ZJSBL01 inoculated group showed clinical symptoms, including less feed intake, green-colored excrements, or neurological sign. Two ducklings of this group died on 5 DPI (Table 2). Three of 10 ducklings in P20 group, 2 of 10 ducklings in P30 group and 2 of 10 ducklings in P50 group showed clinical symptoms, similar with parental ZJSBL01 group, and No clinical symptoms or mortality were observed in the P40-inoculated ducklings (Table 2). ZJSBL01 group ducklings gained less body weight when compared to the uninfected group ducklings in the challenge period, but the body weights of the P20, P30, P40, and P50 group ducklings have no significant difference compared with the mock inoculated group ducklings (Fig. 3A).

The viral RNA amount in the serum of the P20, P30, P40, and P50 groups were significantly lower than that of the parental ZJSBL01 group on 6 DPI (Fig. 3B). Similarly, the viral RNA amount in the liver tissues of the P20, P30, P40, and P50 groups were significantly lower than that of the parental ZJSBL01 group on 6 DPI (Fig. 3C). In addition, the viral RNA amount in the hearts of the P30 and P40 groups were significantly lower than that of the ZJSBL01 group on 6 DPI, and the viral loads in lung of P20 and P40 groups were significantly lower as well (Fig. 3C). These results indicated that parental ZJSBL01 replicates better than P20, P30, P40, and P50 in ducklings.

The necropsies showed that the livers of the ducklings in the P30, P40, and P50 groups were normal on 6 DPI, but the livers of ducklings from the P10, P20 and ZJSBL01 groups had slight bleeding points (Fig. 4A–F). The spleens of the ducklings in the P40 and P50 groups were normal, while the spleens in ducklings from the P10, P20, P30 and ZJSBL01 groups were swollen (Fig. 4G–L). Histopathological observation revealed that the spleen structures were normal in the ducklings from the P40 and P50 groups, whereas the ducklings from the P10, P20, P30 and ZJSBL01 groups showed lymphocyte depletion, endothelial cell exposure, degeneration, and necrosis (Fig. 4M–S). The brain structures of the ducks from the P40 group were normal, whereas the brains of ducklings from the P10, P20, P30, P50 and ZJSBL01 groups showed encephalitis and viral encephalitis (Fig. 4S–Y).

The neutralizing antibodies against DTMUV in the serum of the inoculated or mock inoculated ducklings were determined by competitive ELISA. The result showed that all but one duckling displayed seroconversion at 9 DPI, which indicated that the viruses retained the immunogenicity.

### 3.4. Pathogenicity analysis of P40 in laying ducks

As above results, P40 strain has no virulence for ducklings. Therefore, we evaluated the virulence of P40 for laying ducks. As showed in Fig. 5A, the egg production rate of P40 group was similar with mock group. In contrast, the egg production rate of parental ZJSBL01 group decreased rapidly, and arrived low point on 5 DPI (Fig. 5A). The spleens of ZJSBL01 inoculated ducks showed swollen whereas the P40 and mock inoculated ducks were normal (Fig. 5B–D). No clinical change of ovary was observed in P40 and mock inoculated ducks but severe ovary degenerates in ZJSBL01 inoculated ducks (Fig. 5E–G). In histopathological examination, minimal lymphocytes were observed in the spleen of ZJSBL01 inoculated ducks (Fig. 5J), which also displayed endothelial cell degeneration and necrosis, and

inflammatory cells such as heterophils and lymphocytes observed in the ovaries (Fig. 5T). No change was observed in spleens and ovaries of the mock and P40 inoculated ducks (Fig. 5I and S). These results indicated that P40 strain was attenuated for laying ducks.

### 3.5. Immunogenicity evaluation of different passages of ZJSBL01 in ducklings

P10, P20, P30, P40, and P50 of ZJSBL01 were used to immunize the ducklings for immunogenicity evaluation. The immunized or mock immunized ducklings were challenged with parental ZJSBL01 strain two weeks post immunization. Compared with the mock immunized group, the viral RNA amount of the swabs from the P20 and P40 immunized groups were significantly lower than mock immunized group on 3 day-post-challenge (DPC) (Fig. 6A). And the viral RNA amount of the swabs from the P10, P20, P30, P40 and P50 immunized groups were significantly lower than that from the mock immunized group On 6 DPC, respectively (Fig. 6A).

The viral RNA amount in the hearts, livers and kidneys of all the immunized groups were significantly lower than that of mock immunized group on 3 DPC, respectively. And the viral RNA amount in lungs of P10, P20, P30, and P40 immunized groups were also significantly lower than mock immunized group on 3 DPC (Fig. 6B). The viral RNA amount in the hearts of P10 immunized group, in the livers of P30 and P40 immunized groups, in the spleens of P10, P20, P30, and P40 immunized groups were significantly lower than that of mock immunized group, respectively (Fig. 6B). The viral RNA amount in the hearts of P10 and P20 immunized groups were significantly lower than that of mock immunized group on 6 DPC. The viral RNA amount in the livers of P30 and P40 immunized groups were significantly lower than that of P40 group (Fig. 6C). Compared with the mock immunized group, the viral RNA amount in the Spleens of all except of P50 immunized groups were also significantly lower on 6 DPC (Fig. 6C). In addition, the viral RNA amount in the lungs of P10, P20, and P30 immunized were significantly lower than the mock immunized group (Fig. 6C).

After challenge with virulent ZJSBL01, the necropsies performed on 6 DPC showed that the liver structures of ducklings from the P20, P30, P40, and P50 immunized groups were normal (Fig. 7C–F), whereas the livers of ducks from the P10 immunized group had bleeding points (Fig. 7B). The spleen structure of ducklings from the P40 group was normal (Fig. 7K), and no obvious clinical symptoms were found. In contrast, the spleens of ducklings from the P10, P20, P30, P50 and mock immunized groups were swollen (Fig. 7G–L). Based on histopathological findings, the spleens were normal in the ducklings from the P10, P30, P40 and P50 immunized groups, and no obvious pathological changes were observed, whereas the spleens from the ducklings in the P20 and mock immunized groups showed lymphocyte depletion, endothelial cell exposure, degeneration, and necrosis (Fig. 7M–S). The brain structures of ducklings from the P40 group were normal, but brains of ducks from the P10, P20, P30, P50 and mock immunized groups showed viral encephalitis (Fig. 7T–Y).

## 4. Discussion

DTMUV disease is characterized by egg production dropping and hemorrhagic ovary inflammation in laying ducks, which has brought serious economic losses to the duck industry. Development of an effective vaccine is necessary for prevention and control of the disease. Serial passage attenuation methods are often used for the development of live attenuated flavivirus vaccines (Nitayaphan et al., 1990).

In this study, DTMUV ZJSBL01 strain were attenuated by serial passage in BHK-21 cells in the presence of 5-FU. In the pathogenicity study, all of the birds inoculated with the ZJSBL01 strain displayed clinical symptoms, and two of the ten inoculated birds died during the experiment. Gross lesions of the dead ducklings included cerebral hemorrhage and splenomegaly, which indicated that the ZJSBL01 strain was highly pathogenic to ducklings. In contrast, the P40 caused no morbidity, whereas these occurred in the P20, P30 and P50 inoculated ducklings, respectively. The viral loads in serum, heart, liver, and lung tissues of the P40 inoculated ducklings were lower than that of the ducklings inoculated with virulent ZJSBL01. In addition, the laying ducks inoculated with P40 did not exhibit egg production dropping or clinical changes, whereas ZJSBL01 induced egg production dropping and clinical changes. Therefore, we concluded that the P40 virus was highly attenuated.

Seroconversion was first observed in P40 inoculated ducklings on day 5 post-inoculation, and over 83% (5/6) of them displayed seroconversion on day 9 post-inoculation and all of them displayed seroconversion on day 14 post-inoculation, which indicates that the P40 reserves the immunogenicity. Furthermore, P40 vaccination provided complete clinical protection against parental ZJSBL01 challenge, whereas other passages provided only partial clinical protection in ducklings. After the challenge, the viral loads in almost tissues and swabs of the P40 immunized ducklings were lower than the mock immunized ducklings, which indicated that P40 retained a level of immunogenicity suitable for a DTMUV vaccine candidate.

Many flaviviruses cause serious diseases in human, such as Zika virus, West Nile virus, Japanese encephalitis virus, and Yellow fever virus (Li et al., 2014). Previous study showed that high DTMUV antibody positive rate was detected in the duck farm worker (Tang et al., 2013), and Ti et al. reported the pathogenicity of DTMUV to Kunming mice by intracerebral inoculation (Ti et al., 2016), which suggest the potential public threats of DTMUV. Development of chimeric flaviviruses in which the prM and E genes of a live-attenuated flavivirus vaccine strain are substituted with the corresponding genes of interested flavivirus has widely study for human flaviviruses (Li et al., 2018). The vaccine development against DTMUV plays an important role in the disease control in duck industry and public health. In addition, attenuated DTMUV provides a potential backbone for the development of chimeric vaccine against human flavivirus.

5-FU is a fluoride of pyrimidine, which is widely used for anticancer treatment because of its inhibition of thymidine synthetase (Longley et al., 2003). 5-FU can be used for induced mutation of virus (Pringle et al., 1993), which would shorten the development time of live attenuated vaccine. Nine mutants of cold-passaged respiratory syncytial virus (RSV) were generated which would be satisfactorily attenuated in MRC-5 cells by chemical mutagenesis with 5-FU (Crowe et al., 1994, 1995). In this study, ZJSBL01 strain were continuously passaged in BHK-21 supplied with 5-FU for 50 passages. Different passages were purified by plaque assay to generate P10, P20, P30, P40, and P50 strains. The pathogenicity evaluation showed that P40 is a highly attenuated strain whereas as P50 induced slight pathogenic in ducklings. A reverse mutation 497S was found in P50 compared with P40, which might be due to the plaques selection during purifications. However, whether the 5-FU contributed to the attenuation of ZJSBL01 strain remain further study.

As DTMUV is an economically important virus and has antigenic and biological similarities with other flaviviruses, discovering the molecular mechanism of DTMUV pathogenicity will enrich the knowledge of flaviviruses. Nonetheless, our findings are limited by the predictive nature of our estimation of the contributions of the various mutations observed in the P40 virus with regard to the attenuation of the ZJSBL01 strain of DTMUV. Future reverse genetics studies in animals are guaranteed to elucidate the contributions of the mutated residues to DTMUV pathogenicity.

In summary, we developed an attenuated DTMUV strain, ZJSBL01-P40, by serial passage in BHK-21 supplied with 5-FU. Our results show that ZJSBL01-P40 could be used to prevent DTMUV infection in ducklings. The development of attenuated ZJSBL01-P40 will be helpful to study pathogenicity of flaviviruses and develop vaccines.

## Contributions

FC and JFH designed the research. JFH, HQS and ZXW performed the experiments. JFH, SJH and QHL analyzed the data. QFZ, JPQ and QMX contributed materials/analysis tools; JFH and HQS wrote the manuscript. FC revised the manuscript. All authors read and approved the final version of the manuscript.

## Conflict of interest statement

The authors declare that no contradicts of economic interest.

## Ethics statement

The animal study protocol was approved by the South China Agricultural University Committee of Animal Experiments (approval ID: SYXK-2014-0136). The experiments were closely followed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

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