Adaptation and Attenuation of Duck Tembusu Virus Strain Du/CH/LSD/110128 following Serial Passage in Chicken Embryos

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Duck Tembusu virus (DTMUV) is a newly emerging pathogenic flavivirus that has caused massive economic losses to the duck industry in China. In the current study, a virulent strain of DTMUV, designated Du/CH/LSD/110128, was isolated from the livers of diseased ducks and attenuated by serial passage in embryonated chicken eggs. The virus was partially attenuated after 50 and 70 passages and was fully attenuated after 90 passages, based on mortality and morbidity rates and viral loads in inoculated ducklings. Fourteen amino acid substitutions were observed in the capsid, prM, envelope, NS1, NS3, NS4A, NS4B, and NS5 proteins of the fully attenuated strain of Du/CH/LSD/110128, which might be responsible for the observed changes in replication and pathogenicity. A 72-nucleotide deletion was also observed in the 3’ untranslated region of the virus after 30 passages. The fully attenuated virus retained the immunogenicity of the parental strain, providing effective protection to challenge with virulent Du/CH/LSD/110128, and may represent a suitable candidate as a vaccine strain against DTMUV infection in ducks. Our results also lay the foundation for future studies on the replication and pathogenic mechanisms of DTMUV.

Since April 2010, a severe duck disease has emerged throughout the main duck-producing regions of China. In addition to ducks, the disease has affected geese, chickens, and sparrows (1–3). The infected ducks developed high fever, diarrhea, and anorexia and displayed retarded growth (4). Hyperemia, hemorrhage, degeneration, distortion, and lymphocytic infiltration in the ovaries were the primary pathological features consistently observed in diseased ducks. The disease also caused large decreases in egg production in egg-laying ducks within 1 to 2 weeks postinfection. Based on the clinical signs and pathological features, the disease was designated duck hemorrhagic ovaritis (DHO) (5). The disease is currently circulating in domestic duck flocks in China, and the epidemiology of DHO indicates no seasonality. In addition to the rapid spread among duck populations, DHO might have the potential to infect humans (6, 7), highlighting the need to protect public health.

The etiological agent of DHO was initially identified as a Baiyangdian virus (BYDV) (8). The genome of this etiological agent (the virus) consists of an approximately 10,990-nucleotide (nt), positive-sense, single-stranded RNA with a 7-methylguanosine cap at the 5’ terminus that is flanked by a conserved AG dinucleotide. Lacking a 3’ polyadenylation sequence, the 3’ terminus of the genome consists of a conserved CU dinucleotide. The genome contains one large open reading frame (ORF), within which several genes are arranged in the following order: 5’ untranslated region (UTR), capsid, prM, envelope (E), nonstructural (NS) genes NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, and 3’ UTR (9). Analysis of partial sequences of the E and NS5 genes revealed a close relationship with the Ntaya virus (NTAV) group of the genus Flavivirus. The phylogenetic and antigenic analyses revealed a close relationship to the Tembusu virus and the Sitiawan virus, both of which are members of the genus Flavivirus, family Flaviviridae (8, 10). The virus was independently designated duck Tembusu virus (DTMUV) (11) and Tembusu-like virus of ducks (12).

Given the devastating impact of DHO on duck farming and the threat of transmission to other birds (1, 13), effective control mechanisms for preventing the transmission of DTMUV are needed, among which the development of an effective vaccine would be of particular significance. Effective vaccines for flaviviruses have been developed and widely used for mammals, including those against the yellow fever virus and the Japanese encephalitis virus. Recently, a vaccine candidate against DTMUV passed serially in chicken embryo fibroblasts (14) was reported; however, development of a vaccine against DTMUV by using embryos has not yet been reported.

In the current study, we isolated and propagated a virulent DTMUV strain, designated Du/CH/LSD/110128, in 9- to 11-day-old embryonated duck eggs. The virus was serially passaged 90 times in embryonated chicken eggs. Assessments of viral replication, attenuation of the virus following serial passage, and changes in the nucleotide and amino acid sequences of the virus were the primary objectives of our study, to evaluate the potential of the attenuated virus as a vaccine candidate. Future studies will focus on practical considerations (such as vaccination of meat-type and laying ducks under field conditions) regarding the development of such a vaccine.

MATERIALS AND METHODS

Eggs and ducklings. All of the animals and eggs used in our experiments were specific pathogen free. The fertile duck and chicken eggs and the ducklings used in our experiments were obtained from the Laboratory Animal Center at the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences in the Heilongjiang Province of China.
The birds were maintained in negative-pressure isolators, and food and water were available *ad libitum*. The ducklings were cared for in accordance with the humane procedures and biosecurity guidelines established by the Harbin Veterinary Research Institute.

**Virus isolation and identification.** A virulent DTMUV strain, designated Du/CH/LSD/110128, was isolated from the livers of dead ducks during an outbreak of DHO at a farm in the Shandong Province in China. Severe ovarian hemorrhage, ovaritis, and enlarged spleen were consistently observed in the ducks at necropsy. Ruptured ovarian follicles and peritonitis were also observed in some of the affected ducks. For viral isolation, the liver samples were prepared as 10% (wt/vol) tissue suspensions in 0.1% phosphate-buffered saline (PBS), clarified by centrifugation at 1,500 × g for 10 min at 4°C, and filtered through 0.22-μm membrane filters (Millipore, Bedford, MA) before inoculation into the allantoic cavity of 9- to 11-day-old embryonated duck eggs, and the infectious allantoic fluid was collected 72 h postinoculation (15). The DTMUV strain was identified by reverse transcription (RT) and PCR targeting the 5′-noncoding region of the prM gene (250-bp), using the forward and reverse primers 5′-AGACTGCGTCGTTCCCAGATTCCA-3′ and 5′-CGTCGTTCCCAGATTCCA-3′, respectively. Viral RNA was extracted from 200 μl of Du/CH/LSD/110128 infectious allantoic fluid using TRIzol reagent (Invitrogen, Grand Island, NY), according to the manufacturer’s instructions. The cDNA fragment from viral RNA was amplified and directly sequenced by using the forward and reverse primers. The sequences were used for a BLAST search of the GenBank database, which revealed 98.8% nucleotide similarity with the BYDV (GenBank accession no. JQ920420), suggesting that the newly isolated Du/CH/LSD/110128 strain was a DTMUV. The duck embryo-propagated viral stock of Du/CH/LSD/110128 was produced by inoculating the virus into embryonated eggs via the allantoic cavity and collecting the infectious allantoic fluid 72 h postinoculation, as described previously (15), and was stored at −80°C.

**Adaptation and serial passage of Du/CH/LSD/110128 in chicken embryos.** The Du/CH/LSD/110128 strain was serially passaged 90 times by inoculation into embryonated eggs via the allantoic cavity and was cloned into the pMD18-T plasmid (TaKaRa, Otsu, Japan), according to the manufacturer’s instructions. The copy number of the plasmid was calculated, as described previously (16). The tissue samples collected in experiments 1 and 3 were homogenized, and viral RNA was extracted from 300 μl of each homogenate, as described previously (4). A previously described, TaqMan-based, one-step RT and quantitative real-time RT-quantitative PCR (qPCR) method was used to measure viral loads (17). The RT-qPCR was inoculated with allantoic fluid collected at P0, P50, P70, and P90, respectively, by intracerebral injection of 100 μl of dilute virus stock containing 10^5 times the 50% embryo infectious dose (EID_{50}) of the virus. The birds in group 5 were mock inoculated with sterile allantoic fluid and served as the negative-control group. Three birds from groups 1 to 5 were euthanized on days 3, 7, 14, 21, and 28 postinoculation. Tissue samples were collected from the trachea, lung, liver, spleen, proventriculus, duodenum, small intestine, large intestine, cecum, kidney, rectum, Harderian glands, pancreas, cecal tonsil, bursa of Fabricius, brain, and bone marrow for virus detection.

(ii) **Experiment 2.** Four groups of 1-day-old ducklings were housed in different isolators. Group 1 and the negative-control group contained 10 ducklings each, and groups 2 and 3 contained 8 ducklings each. At 7 days of age, each duckling in groups 1, 2, and 3 was inoculated with Du/CH/LSD/110128 at P0, P70, and P90, respectively, by intracerebral injection of an inoculum containing 10^5 EID_{50} of virus (Table 1). The birds in the negative-control group were mock inoculated with sterile allantoic fluid. The ducklings were examined daily for signs of infection until day 30 postinoculation, and the data were used to assess the pathogenicity of Du/CH/LSD/110128 at P0, P70, and P90. Ducks were exsanguinated on days 5, 10, 15, 20, 25, and 30 postinoculation. Serum samples were stored at −70°C until Western blotting was performed.

(iii) **Experiment 3.** Three groups of 1-day-old ducklings were housed in different isolators. Group 1 contained 18 birds. Group 2 contained 18 birds and was used as the positive-control group. Group 3 contained 10 birds and was used as the negative-control group. At 7 days of age, each bird in group 1 was inoculated by intracerebral injection of 100 μl of P90 virus stock containing 10^5 EID_{50} of virus, and birds in groups 2 and 3 were mock inoculated with sterile allantoic fluid. At 25 days postinoculation, the birds in groups 1 and 2 were challenged by intracerebral injection of 100 μl of pathogenic Du/CH/LSD/110128, and the birds in group 3 were mock inoculated with sterile allantoic fluid. The ducklings in each group were examined daily for signs of infection after challenge. On day 5 postchallenge, nine birds from groups 1 and 2 were euthanized and exsanguinated. On day 10 postinoculation, five birds from group 3 were also euthanized and exsanguinated. Tissue samples were collected from the trachea, lung, liver, spleen, proventriculus, duodenum, small intestine, large intestine, cecum, kidney, rectum, Harderian glands, pancreas, cecal tonsil, bursa of Fabricius, brain, and bone marrow for virus detection.

**Virus detection and quantification.** A 115-nt sequence of the E gene of the virus was amplified by RT-PCR using the forward primer 5′-GTCATATGGGT-3′ and the reverse primer 5′-GTCGTTCCCAGATTCCA-3′, and the sequence was confirmed, and the plasmid was used as the positive-control template for virus detection and quantification. The copy number of the plasmid was calculated, as described previously (16). The tissue samples collected in experiments 1 and 3 were homogenized, and viral RNA was extracted from 300 μl of each homogenate, as described previously (4). A previously described, TaqMan-based, one-step RT and quantitative real-time RT-quantitative PCR (qPCR) method was used to measure viral loads (17). The RT-qPCR was

### TABLE 1 Results of pathogenicity study of Du/CH/LSD/110128 at P0, P70, and P90 in specific-pathogen-free ducklings

| Group | Dose (log_{10} EID_{50}) | No. with morbidity/total no. (%) | No. of mortality/total no. (%) | No. seroconverted/no. inoculated (%) at:
|-------|-------------------------|-----------------------------|-------------------------------|-----------------------------------------------|
|       |                         |                             |                               | 5 days | 10 days | 15 days | 20 days | 25 days | 30 days |
| P0    | 5                       | 10/10 (100)                 | 2/10 (20)                     | 7/9 (77.8) | 7/8 (87.5) | 8/8 (100) | 8/8 (100) | 8/8 (100) | 8/8 (100) |
| P70   | 5                       | 5/8 (62.5)                  | 0/8 (0)                       | 2/8 (25) | 6/8 (75) | 8/8 (100) | 7/8 (87.5) | 6/8 (75) | 6/8 (75) |
| P90   | 5                       | 0/8 (0)                     | 0/8 (0)                       | 1/8 (12.5) | 5/8 (62.5) | 6/8 (75) | 7/8 (87.5) | 8/8 (100) | 7/8 (87.5) |
| Control | 0/10 (0) | 0/10 (0) | 0/10 (0) | 0/10 (0) | 0/10 (0) | 0/10 (0) | 0/10 (0) | 0/10 (0) | 0/10 (0) |

a Dose per duckling.

b Serum samples that had a prominent immunoreactive band (about 66 kDa) in Western blotting were considered positive, and those that did not were considered negative.

c Days after inoculation/challenge.
performed using a One Step PrimeScript perfect real-time RT-PCR kit (TaKaRa-Bio) in a 96-well plate in a LightCycler 480 real-time PCR system (Roche Applied Science, Penzberg, Germany). Thermal cycling was performed at 42°C for 5 min, 95°C for 10 s, and 40 cycles of 95°C for 5 s and 52°C for 30 s. Each assay included at least one negative-control sample containing diethyl pyrocarbonate (DEPC)-treated water in place of the RNA. The size of the RT-qPCR products was confirmed by electrophoresis on a 2.0% agarose gel and ethidium bromide staining. The data were analyzed using the LightCycler 480 (version 1.5; Roche Applied Science) and SPSS (IBM, Armonk, NY) software packages. Statistical analyses, where appropriate, were performed using one-way analysis of variance (ANOVA). Differences with P < 0.05 were considered statistically significant.

Cloning, expression, and purification of recombinant NS1 of Du/CH/LSD/110128. RNA extraction, RT, and PCR amplification of NS1 of Du/CH/LSD/110128 were conducted as described for identification of the virus. The cDNA sequence encoding amino acid residues 1 to 352 of the NS1 protein of the Du/CH/LSD/110128 virus was generated using the primers 5'-GAAGTCGGGTTGCTCAATCGACTTGGC'T-3' and 5'-GCCGCCGGCTTAAACCCCTTGAAACGCA-3'. The sequence was cloned into the pGEX-6P-1 expression plasmid (Invitrogen, Carlsbad, CA) through the EcoRI and NotI restriction enzyme sites, and competent Rosetta Escherichia coli was transformed with the ligation product. Recombinant protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.6 mM. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and staining with Coomassie brilliant blue, as described previously (18). The protein band was excised from the gel. The gel slices were crushed and suspended in an appropriate volume of sterilized PBS for Western blotting, as described previously (19).

Western blotting. Western blotting was performed as described previously (18). Gel-purified recombinant NS1 protein was subjected to SDS-PAGE on a 10% acrylamide gel. The protein band was electrophoretically transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA), and the NS1 protein was detected as described previously (18). The blot was incubated with a 1:200 dilution of a serum sample collected in a pathogenicity study or experiment 3. Antigen-antibody reactivity was detected by incubation with a 1:500 dilution of horseradish peroxidase-conjugated goat anti-duck IgG(H+L) (KPL, Gaithersburg, MD). Serum samples that produced a prominent immunoreactive band corresponding to approximately 66 kDa from the gel were considered to have come from birds that were not infected.

Sequencing of the Du/CH/LSD/110128 genome. Ten pairs of overlapping primers were designed based on the complete genomic sequence of the BYDV (GenBank accession no. JQ920420) (Table 2). Viral RNA was extracted from 200 μl of allantoic fluid collected at P0, P30, P50, P70, or P90, as described previously (15), and was used as a template to generate cDNA for the full-length viral genome. The cDNAs of the 5' and 3' ends of the viral genome were generated by rapid amplification of cDNA ends (RACE) using a 5'/3'RACE kit (TaKaRa-Ra-Bio) with the primers 5'-RACE (5'-CGTGGTTCGAGTTCCA-3') and 3'-RACE (5'-CCACGGATGAA CTAATGGGAA-3'), respectively (20). The cDNAs of the intervening sequences of the genome were generated using a PrimeScript One Step RT-PCR kit (version 2; TaKaRa-Ra-Bio). RT was performed at 50°C for 30 min. Thermal cycling for PCR was performed at 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 50°C for 60 s, and 72°C for 2 min, with a final extension at 72°C for 10 min. The PCR products were subjected to agarose gel electrophoresis, and the DNA was purified from the gel using an OmegaBio-Tek gel extraction kit (Norrenta, CA). The purified DNA products were cloned separately into the pMD18-T plasmid and sequenced.

Nucleotide and amino acid sequence analyses. The sequences obtained in the previous section were manually compiled, and the ORF was determined by using the Gene Runner (version 3.00) program. The ORF sequences were compared with those of BYDV strains. The nucleotide and predicted amino acid sequences for the Du/CH/LSD/110128 genome were assembled and aligned using the MegAlign program (DNAStar, Madison, WI).

Nucleotide sequence accession numbers. The full sequences of DTMUV Du/CH/LSD/110128 at passage 0 (P0), P30, P50, P70, and P90 in this study were deposited in GenBank with the following accession numbers: P0, KCT136210; P30, KJ782377; P50, KJ782378; P70, KJ782379; P90, KJ782380.

RESULTS
Pathogenicity of the Du/CH/LSD/110128 strain. In the pathogenicity study, all of the ducklings exhibited neurological signs on days 3 to 13 postinoculation. The clinical symptoms included lethargy, anorexia, paralysis, opisthotonos, and ataxia. Two ducklings died, at 84 and 132 h postinoculation. Gross lesions were confined primarily to the spleen and brain. Liver hemorrhage and ulcerated proventriculus were also observed in certain affected ducklings, suggesting that the two ducklings succumbed to the infection caused by P0. The clinical signs of infection diminished gradually after 15 days postinoculation. No clinical signs or gross lesions were observed in the negative-control group. The data in Table 1 show that 77.8% and 87.5% of the ducklings challenged with the virulent Du/CH/LSD/110128 strain displayed seroconversion on days 5 and 10 postchallenge, respectively. Seroconversion was observed in all of the birds on day 15 postinoculation.

Du/CH/LSD/110128 was attenuated by serial passage. Lesions, such as edema, were observed in the chicken embryos at P6 and subsequent passages. The data in Table 1 show that 62.5% of the birds inoculated with the P70 allantoic fluid from chicken eggs showed overt pathology, similar to that observed in the birds challenged with the virulent Du/CH/LSD/110128 virus obtained from duck eggs, with clinical signs developing between day 3 and day 10 postinoculation. No deaths, clinical signs, or gross lesions were observed.
TABLE 2 Sequences and positions of oligonucleotides used for RT-PCR

| Primer  | Sequence (5’ to 3’) | Fragment | Product length (nt) | Positions in genome
|---------|---------------------|----------|---------------------|---------------------|
| 1F      | AGACTGCTGGTGCAATGAGAC | F1       | 1,377               | 649–669             |
| 1R      | GTTATCAAAGGTTCACCTGG  |          |                     |                     |
| 2F      | CCACAAACAATCGTGGTG    | F2       | 1,316               | 2006–2025            |
| 2R      | CATATTCCGGGTCTCACAATC |          |                     |                     |
| 3F      | GCGTGGAAATTGTGATGCACTAC | F3    | 1,360               | 1695–1714            |
| 3R      | CAGACAAAGGAGCTAACCC   |          |                     |                     |
| 4F      | GCTTTGGTCCTGTTGAGG    | F4       | 1,362               | 2110–2129            |
| 4R      | TGAATCGGATGTCGTCGTTGC |          |                     |                     |
| 5F      | GAAATACCAGGCTGGAGTGG  | F5       | 1,347               | 2610–2629            |
| 5R      | GGCTGGAATTTGTGACATAC  |          |                     |                     |
| 6F      | GCCGTGCTGGACATGCTTG   | F6       | 1,298               | 3638–3657            |
| 6R      | GCTTCTAACATCCGGCAGAAG |          |                     |                     |
| 7F      | GACAGGCTCTTGAGTATG    | F7       | 1,313               | 4683–4703            |
| 7R      | CTGTCCTGCTCATGCCTGCCG |          |                     |                     |
| 8F      | CAGCTTAATCAACTTAGCCGC | F8       | 1,321               | 5705–5724            |
| 8R      | TGAAATGCAAGGCTGTCACA  |          |                     |                     |
| 9F      | CATCGTGGCAAGATGATGT   | F9       | 1,316               | 6882–6701            |
| 9R      | CCAGACAGCTGTCGAAATGC  |          |                     |                     |
| 10F     | CCAGATGCAACTATGGAGAG  | F10      | 922                 | 7975–7994            |
| 10R     | TCTTCCCTAACCCTCTAGTC  |          |                     |                     |

a F, forward primer; R, reverse primer.
b Positions refer to the complete genome of BYDV (GenBank accession no. JQ920420).

observed in the P90-inoculated ducklings or the negative-control group. Three ducklings challenged with the Du/CH/LSD/110128 strain died on days 3, 5, and 6 postinoculation. All of the birds inoculated with the P50 inoculum displayed neurological symptoms, and one duckling died on day 3 postinoculation. The dead ducklings had gross lesions that were confined primarily to the spleen and brain, which were similar to those observed in ducklings inoculated with virulent virus obtained from duck eggs.

As shown in Table 3, the virus could be detected in all of the tissue samples collected on day 3 postinoculation. The peak viral loads were observed primarily in the spleen, and the viral loads in all of the tissue samples decreased gradually with each passage. Greater decreases in viral loads were observed in the tissues from birds that received the P70 inoculum, compared with the P50 inoculum. The viral loads in all tissues (except for the spleen on day 28 and the brain on day 3 postinoculation) observed with the P90 inoculum were similar to those observed with P70 (Fig. 2).

TABLE 3 Viral distribution in tissues of ducklings inoculated with Du/CH/LSD/110128 at P0, P50, P70, and P90 in pathogenicity study

| Tissue                      | Virus level at:  | 3 days | 7 days | 14 days | 21 days | 28 days |
|-----------------------------|-------------------|--------|--------|---------|---------|---------|
|                             | P0   | P50  | P70  | P90    | P0   | P50  | P70  | P90    | P0   | P50  | P70  | P90    |
| Trachea                     | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Lung                        | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Liver                       | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Spleen                      | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Proventriculus              | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Duodenum                    | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Small intestine             | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Large intestine             | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Cecum                       | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Kidney                      | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Rectum                      | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Harderian glands            | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Pancreas                    | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Cecal tonsil                | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Bursa of Fabricus           | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |
| Brain                       | + +   | + +  | + +  | + +    | + +  | + +  | + +  | + +    | + +  | + +  | + +  | + +    |

a Days postinoculation.
b +++, viral RNA was detected in the tissue samples by the TaqMan real-time RT-PCR assay, and the threshold cycle (Ct) values of the tissue samples ranged from 18 to 24; +, the Ct values of the tissue samples ranged from 24 to 30; +, the Ct values of the tissue samples ranged from 30 to 35; −, no viral RNA was detected in the tissue samples by the real-time RT-PCR assay or the Ct values for the tissue samples were >35.
Virus was not detected in the negative-control group. These results indicated that the P50 and P70 isolates were partially attenuated, whereas the P90 isolate was fully attenuated. Du/CH/LSD/110128 immunogenicity was not altered by serial passage. Serum antibodies specific for DTMUV were measured by Western blotting. The data in Table 1 showed that 25% and 12.5% of the P70- and P90-inoculated ducklings, respectively, displayed seroconversion on day 5 postinoculation, and all of the P90-inoculated ducklings displayed seroconversion on day 25 postinoculation. No deaths or clinical signs were observed in the P90-inoculated ducklings after challenge with virulent Du/CH/LSD/110128. All of the ducklings in the positive-control group showed clinical signs, and one duckling died on day 4 postchallenge. Virus was not detected by RT-qPCR in any of the tissue samples from the P90-vaccinated ducklings after challenge with virulent Du/CH/LSD/110128. In contrast, virus was detected in tissue samples from all of the ducklings in the positive-control group after challenge with the parental strain. The production of anti-Du/CH/LSD/110128 antibodies in the P90-inoculated birds and the absence of clinical symptoms in the P90-vaccinated birds after challenge indicated that the P90 virus was fully attenuated and that it provided protective immunity against the virulent Du/CH/LSD/110128 strain of DTMUV.

Changes in the Du/CH/LSD/110128 genomic sequence after serial passage. Each of the RT-PCRs of the viruses isolated from the P0, P30, P50, P70, and P90 inocula produced a single band of the expected size. Changes in the Du/CH/LSD/110128 genomic sequence that occurred during the 90 serial passages are summarized in Table 4. No sequence changes occurred in NS2B. Two to four silent mutations occurred in the coding regions of the E, NS1, NS2A, NS3, NS4B, and NS5 genes. Most of the nucleotide substitutions in the NS1 and NS3 genes were observed at P30 and thereafter, and all of them resulted in amino acid substitutions. Single-nucleotide substitutions were also observed in the NS3 and NS4 genes at P70 and P90, respectively. One- and two-nucleotide mutations were observed in the 5' and 3' UTRs, respectively, at P30. Single-nucleotide substitutions were observed in the capsid and prM genes at P70. However, these two mutations were observed to have undergone reversion at P90. Single-nucleotide substitutions observed in the NS5 and E genes at P30 and P50, respectively, also underwent reversion during subsequent passages. None of the other aforementioned point mutations underwent reversion. At P30, a 72-nt deletion in the 3' UTR occurred 5 nucleotides downstream of the stop codon (Fig. 3), which remained unaffected in subsequent passages.

DISCUSSION

In our preliminary experiments, we inoculated ducklings with the Du/CH/LSD/110128 strain of DTMUV using intracerebral, intravenous, intramuscular, and intranasal techniques. We found that death and clinical signs, such as neurological symptoms, occurred only in the ducklings inoculated intracerebrally or intravenously; the birds inoculated intracerebrally exhibited higher morbidity and mortality rates than those inoculated via other routes (data...
In addition, the ducklings infected with parental virus and all passages with virulent Du/CH/LSD/110128 developed splenomegaly, and almost all of the dead ducklings inoculated with the P90 inoculum on day 28 postinoculation were slightly higher than those in birds that received the P70 inoculum. In the earlier report, researchers found that the attenuated variant of Japanese encephalitis virus changed the organ tropism, in comparison with the virulent virus (21). The Du/CH/LSD/110128 strain might have changed tissue tropism during serial passage in chicken embryos, although the exact mechanism needs to be investigated further. Similar results showing that viral loads in the brains of ducklings that received the P90 inoculum were higher than those in birds infected with the P70 inoculum at days 3 and 21 postinoculation were also found, but virus was not detectable in the brains of the birds at day 28 postinoculation. Seroconversion was first observed in P90-inoculated ducklings on day 5 postinoculation, and more than 50% of the ducklings displayed seroconversion on day 10. Furthermore, P90 vaccination provided complete clinical protection against challenge with virulent Du/CH/LSD/110128, which indicated that P90 retained a level of immunogenicity suitable for a DTMUV vaccine candidate.

In the genomic sequence analysis, we found that no sequence changes occurred in the NS2B gene and two silent mutations occurred in the NS2A gene, suggesting that NS2A and NS2B genes might not determine the pathogenicity and replication of the Du/CH/LSD/110128 strain. In the dengue virus (DENV), the protease domain of NS3 is located in the N terminus of the protein. The NS3 protease of DENV plays a crucial role in virus replication by cleaving the full-length viral polyprotein into mature functional proteins (22). A previous study showed that amino acid substitutions in the NS3 protein of the tick-borne encephalitis virus caused conformational changes in the active protease domain of the NS2B-NS3 protease complex that influenced enzyme function and virulence (23).

In our current study, mutation of Ile to Met at position 115 in the N terminus of the NS3 protein of the Du/CH/LSD/110128 virus was observed at P70, and the viral loads in the tissues of P70-inoculated ducklings decreased sharply, compared with those induced using previous inocula. Thus, it is possible that the NS3 protein functions in DTMUV replication. In addition, mutation of Leu to Phe occurred at position 6 in the NS4A protein between NS2A and NS2B genes, suggesting that NS2A and NS2B genes might not determine the pathogenicity and replication of the Du/CH/LSD/110128 strain. In the dengue virus (DENV), the protease domain of NS3 is located in the N terminus of the protein. The NS3 protease of DENV plays a crucial role in virus replication by cleaving the full-length viral polyprotein into mature functional proteins (22). A previous study showed that amino acid substitutions in the NS3 protein of the tick-borne encephalitis virus caused conformational changes in the active protease domain of the NS2B-NS3 protease complex that influenced enzyme function and virulence (23).
The interaction of NS1 and NS4A contributes to flavivirus replication (26). We observed mutation of Val to Met at residue 24 in the NS4A protein of Du/CH/LSD/110128 at P30. Thus, the amino acid mutations in the NS1 and NS4A genes might have influenced viral adaptation in chicken eggs by altering the interactions between these proteins.

In the fully attenuated P90 virus, one mutation occurred in the 5' UTR and two mutations and a 72-nt deletion occurred in the 3' UTR. Previous studies have shown that the 5' and 3' UTRs form secondary structures that influence the transcription and translation of the flavivirus genome (19, 27, 28). We were unable to determine whether these changes in the UTRs of the Du/CH/LSD/110128 genome contributed to the attenuation or adaptation of the DTMUV in the chicken embryos. Future studies of DTMUV replication are warranted, to determine the contributions of the 5' and 3' UTRs to virulence and replication.

Because DTMUV is an economically important virus that shares antigenic and biological similarities with other flaviviruses, understanding the molecular mechanisms of DTMUV pathogenicity is an important research objective. Nonetheless, our findings are limited by the predictive nature of our estimation of the contributions of the various mutations observed in the P90 virus with regard to the attenuation of the Du/CH/LSD/110128 strain of DTMUV. Future reverse-genetics studies in animals are warranted to elucidate the contributions of the mutated residues to DTMUV pathogenicity. In addition, other aspects need to be investigated in future studies. First, although intracranial inoculation was used in the present study, other vaccination methods are required to evaluate the vaccine candidate. Second, although vaccination with the P90 virus provided effective protection against the Du/CH/LSD/110128 parental strain, practical investigations of the P90 virus are required to determine whether it might be a suitable candidate for commercial production of an attenuated vaccine strain against DTMUVs, such as studies of the effects of the candidate vaccine on the production performance of egg-laying ducks and the possibility of vertical transmission of the vaccine via eggs. Third, since flaviviruses persist as a mixture of quasispecies, the persistence of wild-type or more-virulent quasispecies in the vaccine strain should also be investigated in future studies. Lastly, experiments related to the ability of the attenuated virus to grow in and to be transmitted by mosquitoes are of importance because most flaviviruses are mosquito or tick borne.

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