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**Supplementary Data**

**Comparative study of the pathogenicity of the mosquito origin strain and duck origin strain of Tembusu virus in ducklings and three-week-old mice**

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**Materials and methods**

**Cells and viruses**

Baby hamster kidney cells (BHK-21) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Shanghai, China) with 10% fetal bovine serum (FBS) (Gibco, New York, USA) and 1% penicillin/streptomycin at 37 °C with 5% CO2.

The TMUV strain rMM\_1775 was rescued on the basis of the full-length cDNA of MM\_1775 (GenBank: JX477685.2) (Wang et al., 2021), which was first isolated from mosquitos in Malaya in 1955. The TMUV strain rCQW1 was rescued by a reverse genetic system using the viral RNAs of CQW1 (GenBank: KM233707.1) (Chen et al., 2018; Guo et al., 2020), which was isolated from ducks in 2013 (Zhu et al., 2015). .

**Animals**

Three-week-old female Kunming mice were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (Sichuan, China). SPF duck embryos were purchased from Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences (Heilongjiang, China) and hatched in the Waterfowl Breeding Center of Sichuan Agriculture University.

**Animal Experiments**

One hundred and two three-week-old female Kunming mice were randomly separated into six groups. Four experimental groups (n = 17 mice per group) were inoculated intracerebrally (i.c.) with 104.5 TCID50 of the rCQW1 and rMM\_1775 viruses at a volume of 50 μL and inoculated intraperitoneally (i.p.) with 104.5 TCID50 of the viruses at a volume of 200 μL. Two mock groups (n = 17 mice) were inoculated with 50 μL and 200 μL DMEM by the i.c. and i.p. routes. The mice were monitored for changes in weight and clinical symptoms for 14 days. Three mice from each group were euthanized at the indicated time points (2, 5 and 8 dpi), and blood and tissue samples from the heart, liver, spleen, lung, kidney, brain, and intestine were collected.

Five-day-old ducklings were randomly divided into three groups: two experimental groups (n = 14 ducklings) and a mock group (n = 14 ducklings). Duckling in the experimental groups were inoculated intramuscularly (i.m.) with 105 TCID50 of the rCQW1 and rMM\_1775 viruses in a volume of 200 μL, and ducklings in the mock group were infected with 200 μL of DMEM. The ducklings were kept in a waterfowl isolator in separate rooms and monitored for changes in weight and clinical symptoms for 14 days. Three ducklings from each group were euthanized at the indicated time points (3, 5 and 7 dpi), and serum and tissue samples from the heart, liver, spleen, lung, kidney, brain, and thymus were collected.

A portion of the mouse and duckling tissue samples was fixed in 10% paraformaldehyde, and the other portion of the tissue samples was stored at -80 °C until use. The serum samples were filtered and stored at −80 °C.

**Detection of Viremia and Viral Load in Tissues**

For detection of viremia, serum samples were 10-fold serially diluted and titrated on BHK-21 cells in 96-well plates. Frozen tissues (0.1–0.15 g) were homogenized in 1 mL of PBS to produce tissue homogenates. Tissue homogenates were clarified by centrifugation and filtration, and the filtrate with 10-fold continuous dilution was titrated for virus infectivity on BHK-21 cells in 96-well plates. The viral titer was calculated by the Karber method on BHK-21 cells as reported (Wang et al., 2021).

**Histopathology** **analysis**

After at least 48 h of fixation in 10% paraformaldehyde, the different tissues were routinely processed and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin (HE) following standard histopathological protocols. The sections were placed on glass slides and dried in an oven at 60 °C for 6 h. Thereafter, the sections were placed into graded alcohol liquid (100%–75%) after deparaffination in xylene. Then, the sections were stained with hematoxylin for 5 min, and hydrochloric acid alcohol was added for differentiation for 10 s. After immersion in eosin alcohol solution for 1 min, the sections were washed with current water, dehydrated using graded ethanol (90%–100%), vitrified by dimethylbenzene and deposited in neutral balsam. The sections were observed under a microscope.

**Statistical analysis**

Statistical analysis was performed with GraphPad Prism 8.0. Data are expressed as the means with standard deviations (SD). Significance was assessed with Student’s *t* test. Viral loads and survival curves were statistically analyzed by two-tailed paired *t* test. A *P* value of < 0.05 indicates statistical significance.

**References**

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Fig. S1. The clinical symptoms of ducklings infected with rCQW1. **A** Paralysis of the lower limbs. **B** Death.