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Electronic Supplementary Material

Construction of an Infectious Clone for Mosquito-Derived Tembusu Virus Prototypical Strain

Xiaoli Wang[1#], Yu He[1#], Jiaqi Guo[1#], Bowen Jiang[1], Mingshu Wang[1, 2], Renyong Jia[1, 2, 3], Dekang Zhu[1, 2, 3], Mafeng Liu[1, 2, 3], Xinxin Zhao[1, 2, 3], Qiao Yang[1, 2, 3], Ying Wu[1, 2, 3], Shaqiu Zhang[1, 2, 3], Yunya Liu[1], Ling Zhang[1], Yanling Yu[1], Anchun Cheng[1, 2, 3]✉, Shun Chen[1, 2, 3]✉

1. Research Center of Avian Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China;
2. Institute of Preventive Veterinary Medicine, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, China;
3. Key Laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu 611130, China

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# Xiaoli Wang, Yu He, Jiaqi Guo contributed equally to this work.

Materials and Methods

Cells and Virus

Baby hamster kidney cells (BHK-21) were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Gibco, New York, USA) and 1% penicillin/streptomycin at 37 °C with 5% CO_2. And duck embryo fibroblast (DEF) cells were cultured in DMEM supplemented with 10% new-born calf serum (NBCS, Gibco) and 1% penicillin/streptomycin incubated at 37 °C with 5% CO_2. *Aedes albopictus* C6/36 cells (gifted by Professor Rui Luo, Huazhong Agricultural University) were cultured in RPMI Medium 1640 basic (Gibco, Beijing, china) supplemented with 10% FBS at 28 °C with no additional CO_2.

DTMUV CQW1 strain (GenBank: KM233707.1) was isolated from the liver tissue of Cherry Valley ducks in southwest China in 2015 (Zhu *et al.* 2015). The positive control rCQW1 was rescued from an infectious clone that contained the full-length cDNA of CQW1. And the complete cDNA was positioned under the control of the T7 promoter elements for *in vitro* transcription (Chen *et al.* 2018).

cDNA Synthesis and Cloning

cDNA fragments (P1, P2, P3) covering the complete genome of MM_1775 were synthesized by Sangon Biotech (Shanghai, China) basing on the MM_1775 sequence from GenBank (ID: JX477685.2). Fig. 1A depicts the scheme to clone and assemble the full genome of MM_1775. Plasmid pACNR (gifted by Professor Jian Yang, North Sichuan Medical College) was used to clone fragments P1, P1–2 and P1–3. And an intron sequence was inserted after nt position 2586 (*NS1* gene). Bacterial strain Top 10 (Invitrogen, California, USA) was used as the *E. coli* host for construction and propagation of cDNA clones. All the cloning procedure followed the manufacturer's protocol using ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). The virus-specific sequence of each intermediate clone was validated by Sanger DNA sequencing before it was used in subsequent cloning steps. The final plasmid containing full-length cDNA of MM_1775 was sequenced to ensure no undesired mutations. A CMV promoter and a SV40 poly (A) signal sequence were engineered at the 5′ ends and 3′ of the complete viral cDNA for eukaryotic transcription. In addition, a hepatitis delta virus ribozyme (HDVr) sequence was engineered at the 3′ ends for generation of the authentic 3′ end of the RNA transcript. All restriction endonucleases were purchased from New England Biolabs (Beijing, China).

DNA Transfection

Plasmid pACNR FL-MM_1775, containing the full-length cDNA of MM_1775, was amplified in *E. coli* Top10 and purified using an Endo-free Plasmid Mini Kit II (Omega, Norcross, GA, USA). For transfection, BHK-21 cells were seeded in a 12-well plate. After 16 h cell culture, the cells were transfected with 1 μg DNA per well using Lipofectamine™ 3000 Transfection Reagent (Invitrogen, California, USA) according to the instructions. After transfection, the cells were incubated at 37 °C with 5% CO_2.

Indirect Immunofluorescence Assay (IFA)

IFA was performed to detect the expression of viral proteins in BHK-21 cells transfected with 1 μg of pACNR FL-MM_1775 plasmid. At 96 h post-transfection, the BHK-21 cells were washed with phosphate buffered saline (PBS) twice and fixed with 4% paraformaldehyde for 1 h at 4 °C. The fixed cells were then permeabilized for 1 h at 4 °C with 0.3% Triton in PBS. After 1 h incubation at 37 °C in a blocking buffer containing 5% bovine serum albumin (BSA) in PBS, cells were treated with primary antibodies (the mouse anti-TMUV polyclonal antibody which is self-prepared) for 2 h and then incubated with goat anti-mouse IgG

conjugated with Alexa Fluor 488 (Thermo Fisher Scientific, Shanghai, China) for 1 h. Finally, cells were stained with DAPI in PBS for 15 min. Each step was followed by washing the cell thrice with ice-cold PBST (1‰ Tween-20 in PBS) for 5 min in an orbital shaker. Fluorescence images were acquired under a fluorescence microscope (Nikon, Tokyo, Japan).

Virus Titration and Plaque Assay

Viral titers were determined by the median tissue culture infectious dose ($TCID_{50}$) method on BHK-21 cells as reported (Chen *et al*. 2018). BHK-21 cells were cultured in 96-well plates. The virus samples were serially diluted 10-fold with DMEM, and then 100 μL of the diluted virus solution was divided into 8 wells on a 96-well plate. After 120 h incubation at 37 °C with 5% CO_2, the presence of viruses was detected by assaying for cytopathic effect (CPE) using microscopy and viral titers were calculated according to the Karber method. The method is as follows, $lgTCID_{50} = L – d (s − 0.5)$, L and d represent the logarithm of the highest dilution and the difference between the logarithm of the dilution, and s represents the sum of the ratio of CPE holes.

For the plaque assay, viral samples were 10-fold serially diluted in DMEM. Each virus dilution was added to BHK-21 cells of ~ 95% confluence in the 12-well plate. DMEM was added into the cells from mock group (200 μL per well). The infected cells were incubated for 1.5 h and gently swirled every 15 min to ensure the complete coverage and infection of monolayer. After the incubation, 1 mL of 0.75% methyl cellulose overlay containing 2% FBS and 1% penicillin/streptomycin was added to each well, and the plate was incubated at 37 °C for 5 days. Then, the methyl cellulose overlay was removed, and the plate was washed twice with PBS, fixed with 10% formaldehyde, and incubated at room temperature for 20 min. After removing the fixative, the plate was stained with 1% crystal violet for 1 min, the cells were washed carefully, and visible plaques were observed.

Growth Curve

BHK-21, DEF and C6/36 cells were seeded in 12-well plates. When the cells reached about 80%, removed the medium, washed with PBS for three times, and then infected with 300 μL rMM_1775 and rCQW1 viruses at a dose of 100 $TCID_{50}$. After 1 h attachment (5% CO_2 at 37 °C or 28 °C), the inocula were removed. The cell monolayers were washed for three times with PBS. Then, 1 mL of DMEM or RPMI 1640 containing 2% FBS or NBCS and 1% penicillin/streptomycin was added to each well. The plates were incubated for three days. The medium was collected per 12 h and subjected to viral titration as described above.

Virulence in Duck Embryos

All duck embryos were purchased from the Waterfowl Breeding Center of Sichuan Agriculture University and were randomly divided into five groups. Five 9-day-old duck embryo eggs per group were injected with 100 μL rMM_1775 or rCQW1 dilution by allantoic cavity inoculation at a dose of 100 and 1000 $TCID_{50}$. And the viruses were diluted to the desired concentration with DMEM. The eggs were continuously incubated at 37 °C and inspected daily with an egg lighter. If the embryo stops moving and the blood vessels shed, the embryo's eggs are considered dead. Record the survival time of the inoculated eggs.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8. Data are expressed as means with standard deviations (SD). Significance was assessed by Student's *t*-test. Virus titers in BHK-21, DEF and C6/36 cells were statistically analyzed by two-tailed paired *t*-test. A P value of < 0.05 indicates statistical significance.

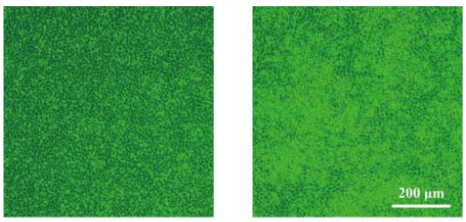

Figure S1. CPE of BHK-21 cells infected rMM_1775 F2. CPE, cytopathic effect.

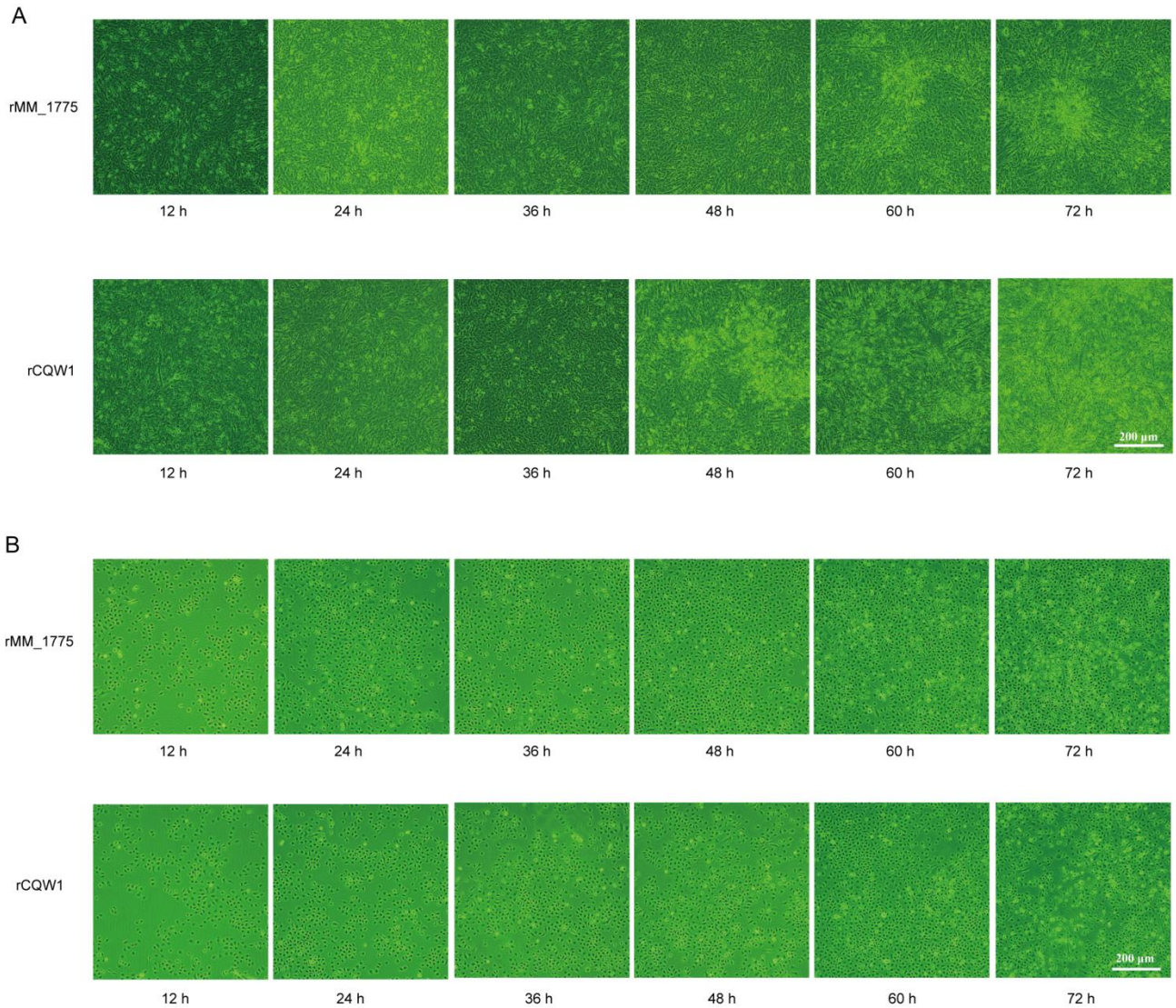

Figure S2. CPE of cells infected with viruses at different time points. A DEFs. B C6/36 cells. CPE, cytopathic effect.

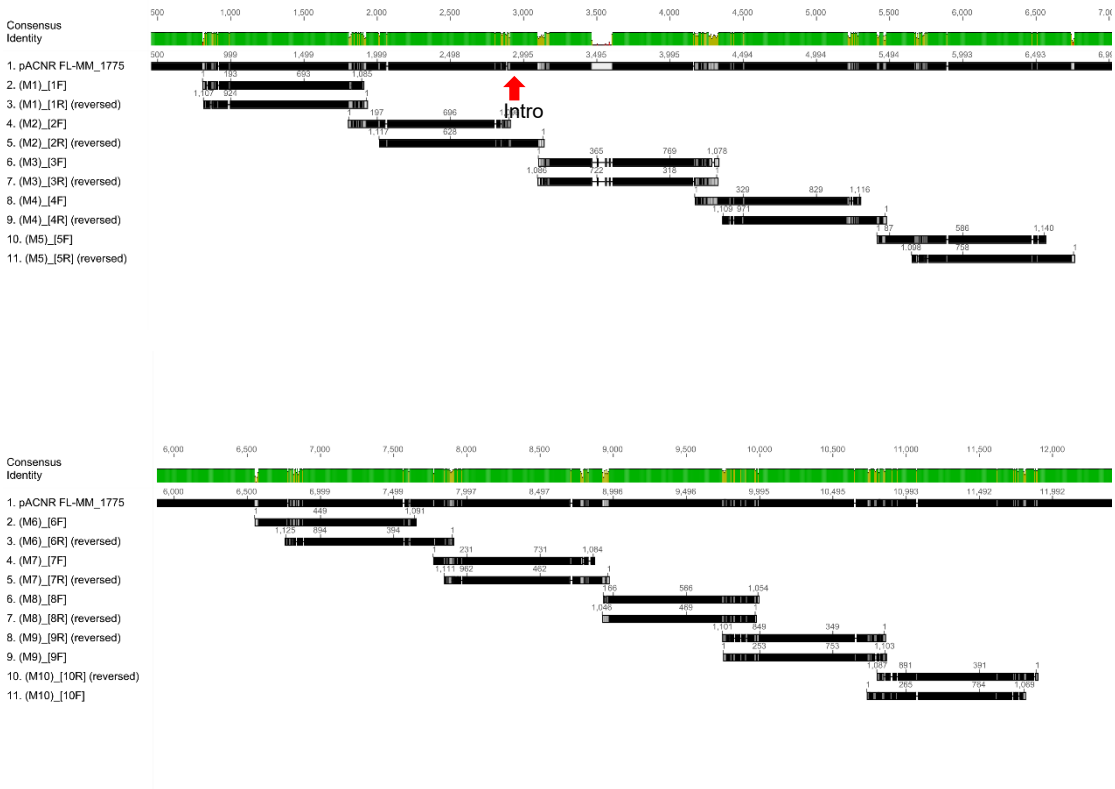

Figure S3. Results of sequencing. The sequence, identified by 10 pairs of primers, was consistent with the plasmid pACNR FL-MM_1775 (GenBank ID: JX477685.2), and there were no mutations. The intron (3459-3591nt, red arrow) is cut off.

The sequences are shown below.

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