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Genetically stable reporter virus, subgenomic replicon and packaging system of duck Tembusu virus based on a reverse genetics system

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ABSTRACT

Duck Tembusu virus (DTMUV) is a novel flavivirus that has caused an outbreak of severe duck egg-drop syndrome since 2010. It has spread rapidly to other avian species, causing enormous economic loss. In the present study, we generated a reporter virus expressing NanoLuc luciferase, which was stable after 10 rounds of continuous propagation without reporter gene deletion. Moreover, we generated two types of replicons driven by the T7 promoter or CMV promoter, both of which worked well in BHK21 cells. Furthermore, we developed the first packaging system for DTMUV by co-transfection into BHK21 cells of a replicon (containing mature C) and a plasmid encoding C_{16}-prM-E, which resulted in the production of single round infectious particles (SRIPs). We also generated a packaging cell line for DTMUV to produce SRIPs. We believe that these multicomponent platform tools are important for DTMUV pathogenesis research and novel vaccine development.

1. Introduction

Duck Tembusu virus (DTMUV) is a novel flavivirus within the family *Flaviviridae* (Su et al., 2011). The *Flavivirus* genus consists of more than 70 viruses, most of which are arthropod-borne viruses (i.e., arboviruses) that are transmitted to their vertebrate hosts by either mosquitoes or ticks. Many flaviviruses, such as dengue virus (DENV), zika virus (ZIKV), yellow fever virus (YFV), Japanese encephalitis virus (JEV), west Nile virus (WNV), and tick-borne encephalitis virus (TBEV), pose a significant threat to public health worldwide. Flaviviruses are enveloped viruses that have a positive-strand RNA genome of approximately 11000 nucleotides. The genome contains a single ORF surrounded by a 5′UTR and 3′UTR, encoding a long polyprotein. This polyprotein is co- and post-translationally processed by host cell signalases and viral NS3 protease (with the cofactor NS2B), giving rise to 3 structural proteins (C, prM, E) to form viral particles and 7 nonstructural proteins (NS1, NS2A/2B, NS3, NS4A/B, NS5) to form replication complexes.

DTMUV infection can cause severe egg-drop syndrome in ducks (Su et al., 2011). Since the first outbreak in 2010, it has led to a serious economic loss for the Chinese poultry industry. DTMUV exhibits a wide range of natural infection hosts, including mosquitoes and various avian species (Su et al., 2011; Zhang et al., 2017). In addition, DTMUV replicates well in many mammalian cell lines (Wang et al., 2016) and showed mouse neurovirulence in a laboratory infection study (Ti et al., 2016). Considering the zoonotic nature of flaviviruses, it is necessary to pay attention to the evolution of DTMUV and its risk of infection in humans. Although the host immune response for DTMUV (He et al., 2017) and other aspects have been broadly investigated, the molecular mechanism of the cross-species transmission of DTMUV and its pathogenicity remains unclear.

In the current study, we generated a genetically stable reporter virus expressing a NanoLuc luciferase (NLuc), and both T7 promoter-driven and CMV promoter-driven subgenomic replicons were constructed and worked well in BHK21 cells but not in DEF cells. Furthermore, we developed the first packaging system based on a packaging cell line, resulting in the production of single round infectious particles (SRIPs). In summary, we developed multicomponent platforms for DTMUV based

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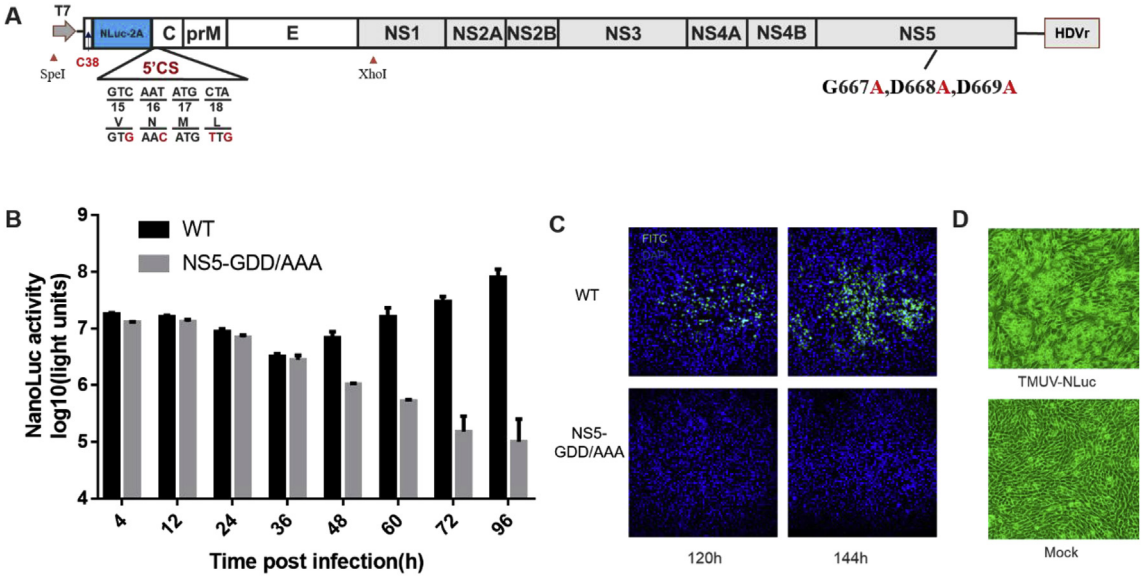

Fig. 1. Construction of reporter DTMUV expressing NanoLuc luciferase. (A) The schematic diagram for the construction of DTMUV-NLuc. C38 indicates the first 38 aa of the C protein, and 2A indicates the FMDV 2A peptide. (B) Luciferase activity kinetics of BHK21 cells transfected with DTMUV-NLuc RNA. Each data point represents the average and standard deviation (SD) from triplicate samples. (C) At 120 h and 144 h post-transfection with DTMUV-NLuc-WT RNA, strong green fluorescence was detected by IFA using mouse anti-TMUV polyclonal antibody as the primary antibody. (D) BHK21 cells appeared CPE at 200 h post-transfection.

on a reverse genetics system, covering viral proliferation, replication, assembly/release and entry. These tools, together with our previously reported infectious clone (Chen et al., 2018), are essential to research on DTMUV disease outbreaks, pathogenesis and vaccine development. More importantly, our study advances the development of flavivirus reverse genetics technologies.

2. Materials and methods

2.1. Cells

Baby hamster kidney cells (BHK21) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Shanghai, China) supplemented with 10% foetal bovine serum (FBS) (Gibco, New York, USA) and incubated at 37 °C with 5% CO_2. Duck embryonic fibroblasts (DEF) cells were cultured in DMEM supplemented with 10% newborn calf serum (NBCS) (Gibco) and incubated at 37 °C with 5% CO_2.

2.2. Reporter virus and replicon plasmid construction

The design scheme of the reporter virus DTMUV-NLuc is shown in Fig. 1A. The DTMUV full-length cDNA infectious clone pACYC FL-TMUV (Chen et al., 2018) was used as the backbone. To engineer a NanoLuc luciferase gene into the DTMUV genome, a series of overlapping PCRs were performed. The luciferase gene was fused in frame downstream of the first 38 amino acids (aa) of the vial capsid gene, and foot-and-mouth disease virus (FMDV) 2A peptide (20 aa in length) was engineered to enable cleavage between the C terminus of luciferase and the N terminus of the downstream capsid protein. Silent mutations within the 5′ native cyclization sequence (5′CS, located in amino acids 15–18 of capsid protein) in full-length capsid were engineered to limit long-range interactions with the 3′CS. A hepatitis delta virus ribozyme (HDVr) sequence was engineered at the 3′ ends of the complete viral cDNA to generate the authentic 3′ end of the RNA transcript of the virus. The full-length cDNA infectious clone for DTMUV-NLuc was designated pACYC DTMUV-NLuc.

We designed two replicons carrying different promoters, as shown in Fig. 3A. The first replicon (CMV-Replicon-NLuc) was driven by a CMV promoter, so the CMV-Replicon-NLuc plasmid can be directly transfected into cells without *in vitro* transcription, and an SV40 polyadenylation signal sequence was engineered for transcription termination. The second replicon (Replicon-NLuc) was driven by a T7 promoter. A large proportion of viral structural protein was replaced by a NanoLuc luciferase gene through a series of overlapping PCRs, while the C protein's first 38 aa and the E protein's last 30 aa were retained in both replicons. Defected replicons with inactivated NS5-GDD motifs were generated simultaneously, as shown in Fig. 3A.

2.3. RNA transcription and transfection

The RNA transcription procedure was performed as previously reported (Chen et al., 2018). Plasmids were purified using an Endo-free Plasmid Mini Kit II (Omega Bio-tek, Georgia, USA) and linearized with the restriction enzyme *Not*I or *Sma*I (NEB, Beijing, China). For transfection, BHK21 cells were seeded in 12-well plates. After 16 h of cell culture (70–90% confluence), cells were transfected with 1 μg RNA per well using Lipofectamine MessengerMAX reagent (Invitrogen, CA, USA) according to the manufacturer's instructions. After transfection, the cells were incubated at 37 °C with 5% CO_2. For DTMUV-NLuc, the supernatant was not harvested until an obvious cytopathic effect (CPE) appeared, and then it was used for the next infection.

2.4. Indirect immunofluorescence assay (IFA)

IFA was performed as previously reported (Chen et al., 2018). Briefly, cells were washed with phosphate-buffered saline (PBS) twice, fixed with 4% paraformaldehyde for 1 h at 4 °C, and 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% bull serum albumin (BSA) in PBS, cells were treated with primary antibodies 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).

2.5. Virus titration, growth curve and luciferase curve

Viral titres were determined by the median tissue culture infectious dose ($TCID_{50}$) method on BHK21 cells as previously reported (Chen et al., 2018). The viral sample was serially diluted 10-fold in DMEM, and then 100 μL dilutions of the viral sample were distributed to each of 8 wells of a 96-well plate seeded with monolayer BHK21 cells. After 120 h incubation at 37 °C with 5% CO_2, the presence of viruses was detected by assaying for CPE using microscopy, and viral titres were calculated according to the Karber method.

The experimental procedure for measuring the viral growth curve was the same as described (Chen et al., 2018) with slight alteration. BHK21 cells and DEF cells were seeded in 24-well plates, and the cells were infected with DTMUV-NLuc at 250 $TCID_{50}$. Every 12 h, supernatant was collected and subjected to viral titration as described above. Simultaneously, after the supernatant was harvested, the cell monolayer was washed once with PBS and then lysed using Glo lysis buffer (Promega, WI, USA) at room temperature for 5 min. The cells were scraped from the plates and stored at −80 °C for the next luciferase activity assay.

2.6. Luciferase activity assay

To detect NLuc activity, a Nano-Glo Luciferase Assay System (Promega) and a GloMax Navigator System (Promega) were used according to the manufacturer's instructions. Then, 100 μL of Nano-Glo Luciferase Assay Reagent was added to 20 μL of the sample in a white 96-well tissue culture plate. The solution was mixed for optimal consistency, and then luminescence was detected.

2.7. Packaging system

To construct an efficient packaging system for DTMUV, a DNA-based replicon (mC-Replicon-NLuc) expressing mature capsid protein (aa 1–104) was designed as shown in Fig. 4A, and aa 1–109 of capsid were engineered to ensure mature C was completely cleaved by NS2B3. Plasmid pCDNA3.1-C_{16}prME encoding the polyprotein C_{16}-prM-E was transfected into cells to supply prM and E proteins *in trans*, forming viral particles. When BHK21 cells seeded in a 12-well plate were at 70–90% confluence, equal amounts of mC-Replicon-NLuc and pCDNA3.1-C_{16}prME plasmids were co-transfected into BHK21 cells using TransIntro EL Transfection Reagent (TransGen Biotech, Beijing, China) per the manufacturer's instructions. After transfection, the cells were incubated at 37 °C with 5% CO_2 for up to 4 days. The DTMUV-SRIPs in the supernatant were harvested every day, aliquoted, and stored at −80 °C.

To verify the packaging efficiency, BHK21 cells were seeded in 48-well plates, and when the cells were at approximately 90% confluence, the cell culture medium was removed and washed three times with PBS. Then, the cells were infected with 100 μL DTMUV-SRIPs and incubated at 37 °C with 5% CO_2 for 2 h. Afterwards, the supernatant was removed and replaced with cell culture maintenance medium (DMEM with 2% FBS and 1% penicillin/streptomycin). Twenty-four hours post-infection, the cells were lysed for luciferase activity assay as described above.

2.8. Packaging cell lines

To establish cell lines constitutively expressing C_{16}-prM-E, 70–90%-confluent BHK21 cells in T25 were transfected with 10 μg pCDNA3.1-C_{16}prME plasmid using Lipofectamine 3000 (Invitrogen). At 24 h post-transfection, G418 (Solarbio life sciences, Beijing, China) was added at a final concentration of 400 μg/mL for selection. The culture medium was replaced with fresh medium containing 400 μg/mL G418 every 3 days until G418-resistant colonies were formed. Then, the cells were digested and diluted in DMEM containing G418 and seeded in 96-well plates. Monoclonal cells were selected and further expanded. Cells were characterized by IFA as described above.

3. Results

3.1. Recovery and identification of DTMUV-NLuc

In a previous study, we established an efficient reverse genetics system for the clinical DTMUV strain CQW1. On the basis of the full-length cDNA clone, a cassette containing the NanoLuc luciferase gene was amplified by a series of overlapping PCRs and ligated with linearized pACYC FL-TMUV using the *Spe*I and *Xho*I restriction enzyme sites. The full-length cDNA infectious clone (pACYC DTMUV-NLuc) for DTMUV-NLuc was successfully constructed. Simultaneously, a mutant clone (pACYC DTMUV-NLuc-NS5-GDD/AAA) with an inactivated NS5-GDD motif was generated. The fidelity of sequences was verified by Sanger sequencing to ensure that there was no undesirable mutation. All plasmids were expanded at 25 °C and 140 rpm.

To recover DTMUV-NLuc, 70–90%-confluent BHK21 cells were transfected with RNA transcripts obtained from *in vitro* transcription. At 4 h post-transfection, the RNA transcripts of both DTMUV-NLuc-WT- and DTMUV-NLuc-NS5-GDD/AAA-transfected cells generated robust luciferase signals. At 36 h post-transfection, the luciferase activities of DTMUV-NLuc-WT-RNA increased over time (Fig. 1B), indicating viral replication. The fluorescence signal detected by IFA also increased from 120 h to 144 h post-transfection (Fig. 1C). However, the luciferase generated by DTMUV-NLuc-NS5-GDD/AAA-RNA remained at a very low level over time, and no fluorescence signal was detected by IFA at 120 h or 144 h post-transfection (Fig. 1B and C). Approximately 200 h post-transfection, clear CPE was observed in BHK21 cells transfected with TMUV-NLuc-WT-RNA (Fig. 1D). Furthermore, the results of whole-genome sequencing of DTMUV-NLuc (round 0) indicated that there was no mutation (data not shown). These data indicate that DTMUV-NLuc was successfully rescued.

3.2. Characteristics of DTMUV-NLuc

DTMUV-NLuc was continuously propagated in BHK21 cells. We determined the viral growth kinetics and the luciferase kinetics in cell cultures using DTMUV-NLuc-F1. As shown in Fig. 2A, titres of DTMUV-NLuc-F1 in BHK21 cells were significantly higher than those in DEF cells at the same time points from 12 to 48 h post-infection and reached a peak ($10^{5.33}$ $TCID_{50}$/100 μL) at 48 h post-infection. Moreover, DTMUV-NLuc-F1 showed very robust luciferase activities in BHK21 cells, reaching a peak ($10^{8.6}$ units) at 48 h post-infection (Fig. 2B). By contrast, DTMUV-NLuc-F1 showed modest growth kinetics and luciferase kinetics in DEF cells (Fig. 2A and B), and both viral titres and luciferase activities were increased over time at the timepoints checked. These data indicate that DTMUV-NLuc was adopted in BHK21 cells better than in DEF cells.

To assess the stability in cell cultures, DTMUV-NLuc was continuously propagated in BHK21 cells, and then F1 ~ F10 of DTMUV-NLuc were subjected to RT-PCR. As shown in Fig. S1, the luciferase gene was still inserted in the viral genome after propagation for 10 rounds, indicating that DTMUV-NLuc was stable after 10 propagations in BHK21 cells. Whole-genome sequencing of DTMUV-NLuc-F1 ~ F5 and F10 revealed 3 adaptive mutations (NS5-G643R, 3′UTR-C215T, E-Q392R) in addition to the original inserted cassette in F3 ~ F10. These data demonstrate that the inserted marker of DTMUV-NLuc was stable in BHK21 cells but that DTMUV-NLuc adapted in BHK21 cells quickly.

3.3. Construction and characteristics of subgenomic replicon

Fig. 3A depicts the DTMUV subgenomic replicons containing a NanoLuc luciferase. Similar to the construction of the reporter virus, a NanoLuc gene was engineered into the full-length cDNA infectious clone (pACYC FL-TMUV) to replace a large portion of the viral

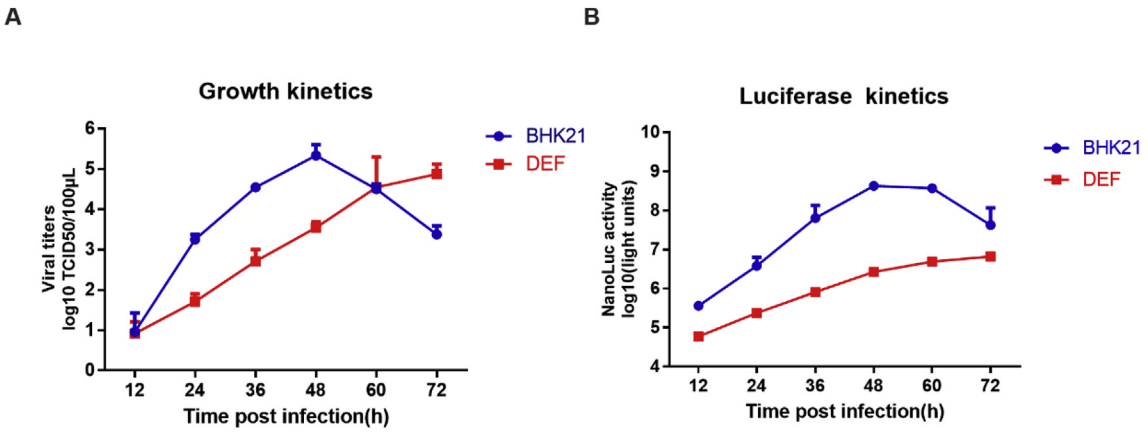

Fig. 2. Growth characteristics of DTMUV-NLuc. (A) Growth kinetics of DTMUV-NLuc in infected BHK21 cells and DEF cells. (B) Luciferase activity kinetics of TMUV-NLuc in infected BHK21 cells and DEF cells. Each data point represents the average and SD from triplicate samples.

structural protein, generating replicon-NLuc (T7 promoter). To construct a replicon with the CMV promoter, a CMV promoter and SV40 poly(A) signal sequence were cloned into the pACYC177 plasmid using the *Nhe*I and *Cla*I restriction enzyme sites, generating pACYC177B, and CMV-Replicon-NLuc was generated via subcloning using replicon-NLuc as the template and pACYC177B as the vector.

Upon transfection into BHK21 cells, the luciferase activity kinetics curve was determined, and both wild-type (WT) replicons with different promoters generated two distinct NLuc peaks (Fig. 3B and C). The first peak was generated by translation of the replicon, and after a period of slump, a more powerful peak was generated by the replication of the subgenomic replicon. As negative controls, both replicons (driven by different promoters) with an inactive NS5-GDD/AAA motif (NS5-GDD/AAA) abolished the second peak but did not affect the first peak. These results are in agreement with the previously reported flavivirus replicons (Xie et al., 2016). Unexpectedly, when replicon assays were performed in DEF cells, no second peak of luciferase activity kinetics was detected (Fig. S2). Collectively, these results indicate that subgenomic replicons for DTMUV were constructed successfully.

3.4. Packaging system

Using the replicon-NLuc, we attempted to construct a packaging system for DTMUV to produce SRIPs for a viral packing study. Several studies have indicated that expression of prM-E of flaviviruses results in the assembly of viral-like particles (VLPs) (Li et al., 2018; Yamanaka et al., 2014). First, we used the Semliki forest virus (SFV) replicon (SFV1-TMUV-CprME) expressing structural proteins of DTMUV via the subgenomic promoter of SFV as previously reported in ZIKV (Mutso et al., 2017). BHK-21 cells were co-transfected with DTMUV replicon-NLuc RNA, and SFV1-DTMUV-CprME RNA resulted in very modest efficiency of SRIPs production because of the limited efficiency of transfection (data not shown). Then, we constructed a new replicon, mC-Replicon-NLuc, carrying mature capsid protein of DTMUV driven by a CMV promoter and plasmid pCDNA3.1-C_{16}prME encoding the C_{16}-prM-E polyprotein (Fig. 4A). Two plasmids were co-transfected into BHK21 cells, and the supernatant was harvested every day and used for next-round infection (experimental flowchart as shown in Fig. 4B). As shown in Fig. 4C, robust NLuc activity was detected and reached a peak ($10^{5.4}$ units) at 3 days post-infection. These data demonstrate the successful formation and release of DTMUV-SRIPs in BHK21 cells and the entry of DTMUV-SRIPs into infected BHK21 cells.

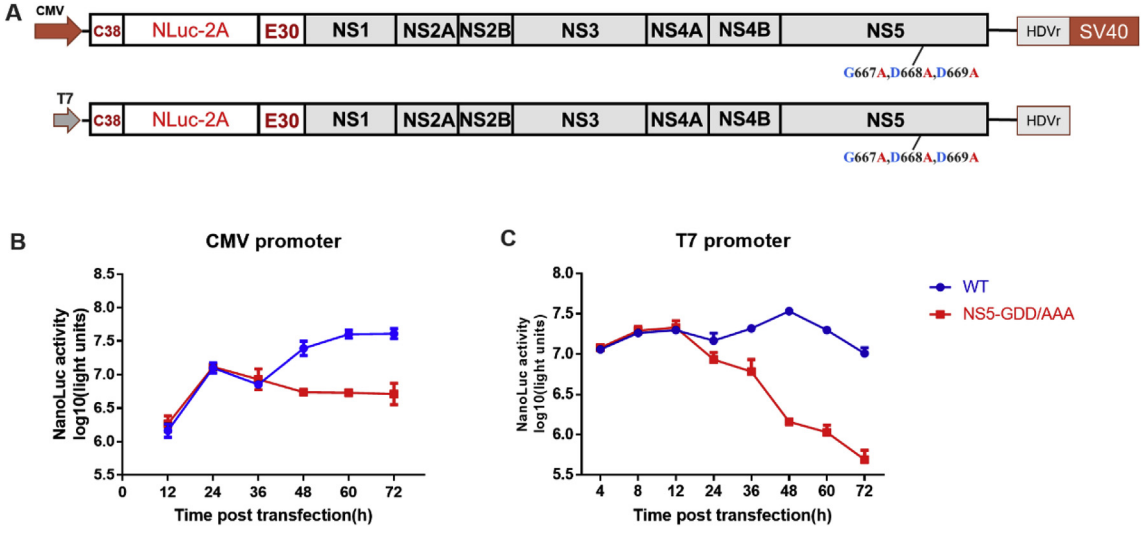

Fig. 3. Construction and characteristics of DTMUV subgenomic replicon. (A) The schematic diagram for the construction of replicons: CMV-Replicon-NLuc (above) and Replicon-NLuc (below). E30 indicates the last 30 aa of the E protein. (B) Replicon assay for CMV-Replicon-NLuc: luciferase activity kinetics of transfected BHK21 cells. (C) Replicon assay for Replicon-NLuc: luciferase activity kinetics of transfected BHK21 cells. Each data point represents the average and SD from triplicate samples.

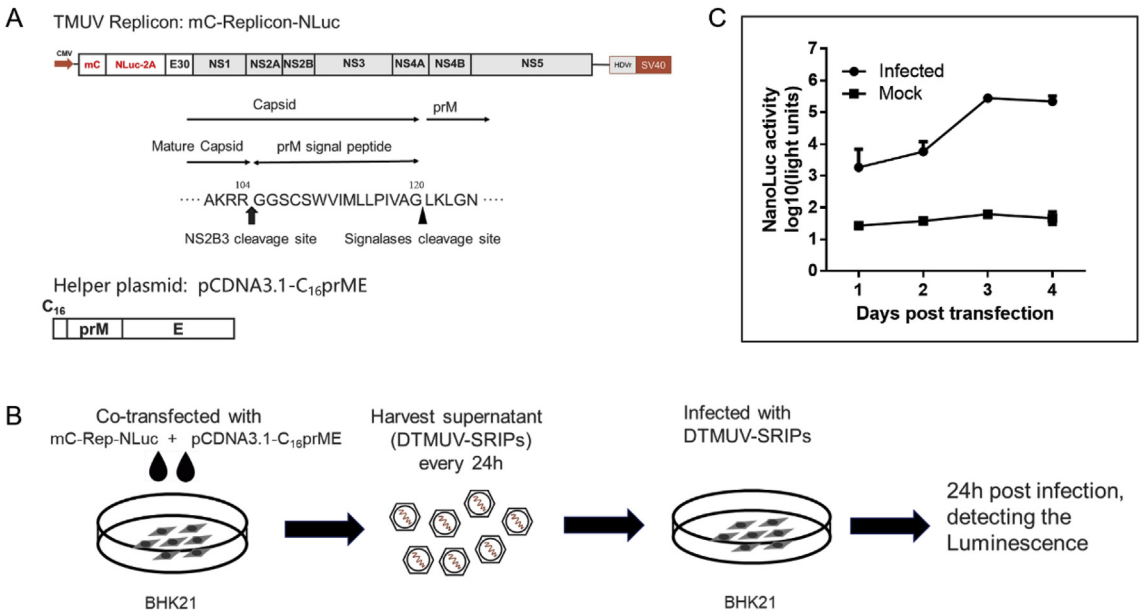

Fig. 4. Construction of a DNA-based DTMUV packaging system. (A) A schematic diagram for the construction of mC-Replicon-NLuc and pCDNA3.1-C_{16}prME. (B) Schematic presentation of the experimental procedure used for the production of SRIPs. (C) Luciferase activity kinetics of SRIP (harvested as described in B)-infected BHK21 cells. Each data point represents the average and SD from triplicate samples.

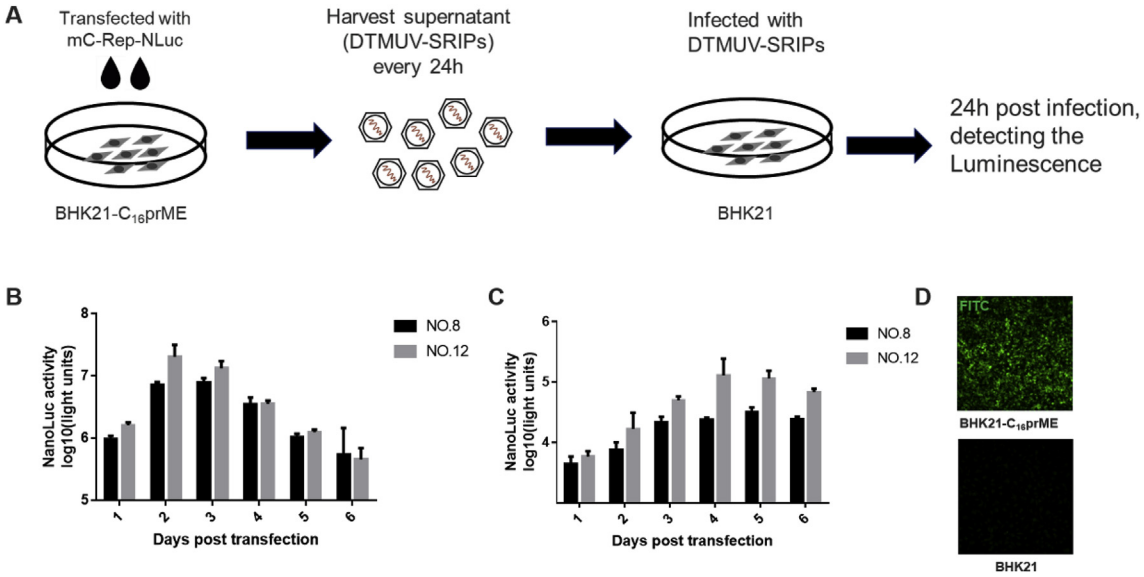

Fig. 5. DTMUV packaging BHK21 cell line. Two strains of a packaging cell line (No. 8 and No. 12) were individually transfected with mC-Replicon-NLuc to produce SRIPs. (A) Schematic presentation of the experimental procedure for SRIPs using a packaging cell line. (B) Luciferase activity kinetics of replicon in two packaging cell lines. (C) Luciferase activity kinetics of SRIPs (harvested as A described) infected BHK21 cells. Each data point represents the average and SD from triplicate samples. (D) The expression of prM or E proteins in packaging cells was detected by IFA using mouse anti-TMUV polyclonal antibody as the primary antibody.

To facilitate convenient applications of these vectors in the production of SRIPs, we next established a packaging cell line, BHK21-C_{16}prME, using pCDNA3.1-C_{16}prME, which stably expressed the polyprotein C_{16}-prM-E. Two strains of monoclonal cells with relatively high expression (identified by IFA) were ultimately selected and further expanded. After continuous passage for 10 rounds, the cells were transfected with the mC-Replicon-NLuc plasmid. The NLuc activity was monitored every day, and the supernatant was harvested simultaneously and used for the next infection. As shown in Fig. 5B, mC-Replicon-NLuc replicated better in BHK21-C_{16}prME-NO.12 than in BHK21-C_{16}prME-NO.8. Corresponding to the results of the replicon assay above, BHK21 cells infected with SRIPs produced by BHK21-C_{16}prME-NO.12 generated significantly more powerful NLuc activity than did those produced by BHK21-C_{16}prME-NO.8 (Fig. 5C). The expression of C_{16}-prM-E in the stable cell line was verified by IFA (Fig. 5D). Taken together, these data demonstrate the successful production of SRIPs with DNA-based DTMUV replicons and the establishment of a packaging cell line for DTMUV.

4. Discussion

Reverse genetics systems have proven valuable in the past few decades and are one of the most powerful tools in molecular virology research. In a previous study, we established an efficient reverse genetics system for the clinical strain CQW1 and generated a *Renilla* luciferase-expressing reporter virus (TMUV-RLuc) (Chen et al., 2018).

Although TMUV-RLuc worked well in antiviral assays, it was unstable in continuous five passages, therefore limiting its application. This prompted us to improve our experimental design to generate a more stable reporter virus. In the present study, we used NanoLuc luciferase as a reporter protein, which is smaller but brighter than *Renilla* and firefly luciferase. In addition, NanoLuc luciferase possesses a number of physical properties that make it an excellent marker for the construction of reporter viruses. Indeed, our data demonstrate that NanoLuc-expressing reporter virus (DTMUV-NLuc) generated a very forceful peak of luciferase activity (Fig. 2B), and the inserted marker was quite stable for at least 10 rounds of propagation (Fig. S1), outclassing our previously reported TMUV-RLuc (Chen et al., 2018).

The CQW1 strain was isolated from clinical duck samples (Zhu et al., 2015), and our previous data demonstrate that it replicates better in avian cells than in BHK21 cells (Chen et al., 2018). However, in the present study, DTMUV-NLuc showed a more robust viral growth curve (Fig. 2A), and the luciferase kinetics (Fig. 2B) in BHK21 cells, rather than DEF cells, deviated from the rule. One possibility is that DTMUV-NLuc was adopted by BHK21 cells early in the first passage. The results of whole-genome sequencing revealed an adaptive mutation in DTMUV-NLuc-F1 as NS5-G643R. However, further experimental data are needed to demonstrate whether this single mutation in CQW1 NS5 is responsible for the change in viral replication in BHK21 cells and ultimately results in better viral propagation. Given the rapid adaptation of DTMUV-NLuc in BHK21 cells, other mutations may appear during the detection process of viral replication kinetics and could be responsible for the results.

Subgenomic replicon assays are widely used in research on flaviviruses, such as DENV (Kato and Hishiki, 2016), ZIKV (Xie et al., 2016) and JEV (Li et al., 2016). Because a large proportion of structural proteins are deleted, replicon assays do not generate infectious particles, reducing the requirement for biological safety and eliminating the risk of laboratory infection. In addition, these assays cover aspects of viral translation and replication but do not include aspects of viral entry or virion assembly/release, which makes them a convenient and practical tool for research on the mechanism of viral replication and screening of antivirals. In the present study, we used two strategies for replicon constructs with different promoters. Replicon-NLuc plasmids under the control of the T7 promoter need to be transcribed *in vitro* before use. BHK21 cells transfected with RNA transcripts directly result in replication of the subgenomic RNA. In this stage, the RNA-based replicon assay mimics viral replication. CMV promoter-driven replicon constructs allow direct transfection of plasmid, but RNA transcript production might be interfaced by splice sites in viral subgenomes when using this approach (Hilgenfeld and Vasudevan, 2018). Nevertheless, this strategy has been pursued successfully in our study (Fig. 3B). Quite unexpectedly, the replicon assay failed in DEF cells. The replicon did not replicate at all after initial translation, even after 3 experiments (Fig. S2), and the reason behind this is still unknown.

Here, we reported the first packaging system for DTMUV to establish a research platform covering viral assembly/release and entry, which could generate SRIPs packed with RNA of the viral subgenomic replicon. This system will be useful for viral mutagenesis studies, such as antiviral discovery (Lu et al., 2017) and vaccine development (Chang et al., 2008). In this platform, mature capsid protein was kept in the DNA-based subgenomic replicon construct (Fig. 4A) to carry viral subgenomic RNA into VLPs, C_{16}-prM-E was supplied *in trans*, so it is very easy to make later modifications in mutational studies of viral structural proteins and nonstructural proteins. In addition, the efficiency of the system could be improved by optimizing the transfected ratio of the two plasmids and efficient transfection procedures. We also generated a packaging cell line for DTMUV to produce SRIPs, but the efficiency still needs to be improved (Fig. 5C) because of its low protein expression efficiency. To improve the virion production of packaging cell lines, a tetracycline-inducible expression system might be a better choice (Harvey et al., 2004). It should be noted that in addition to SRIPs, there are subviral particles (SVPs) in the supernatant of transfected cells because of excess prM-E proteins. The SVPs did not contain capsid or viral subgenomic RNA but increased the overall antigen production in the supernatant, which should be taken into consideration in some applications.

In conclusion, the current study developed powerful platforms for DTMUV based on a reverse genetics system, covering viral translation, replication, assembly/release and entry. We generated a reporter virus expressing a NanoLuc luciferase, which was stable for 10 rounds of continuous propagation and adapted quickly in BHK21 cells. Both T7 promoter-driven and CMV promoter-driven replicons worked well in BHK21 cells but not in DEF cells, indicating that BHK21 cells are a perfect cell model for research on duck TMUV. Furthermore, we developed the first packaging system for DTMUV, involving co-transfection of a replicon and a plasmid encoding C_{16}-prM-E, resulting in the production of SRIPs. We also generated a packaging cell line for DTMUV, although the efficiency needs to be improved. These tools, together with our previously reported infectious clone, provide a critically multicomponent platform for research on DTMUV disease outbreaks, pathogenesis and vaccines.

Conflicts of interest

The authors declared that there are no competing financial interests regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.05.003.

References

Chang, D.C., et al., 2008. Single-round infectious particles enhance immunogenicity of a DNA vaccine against West Nile virus. Nat. Biotechnol. 26 (5), 571–577.
Chen, S., et al., 2018. Establishment of a reverse genetics system for duck Tembusu virus to study virulence and screen antiviral genes. Antivir. Res. 157, 120–127.
Harvey, T.J., et al., 2004. Tetracycline-inducible packaging cell line for production of flavivirus replicon particles. J. Virol. 78 (1), 531–538.
He, Y., et al., 2017. Differential immune-related gene expression in the spleens of duck Tembusu virus-infected goslings. Vet. Microbiol. 212, 39–47.
Hilgenfeld, R., Vasudevan, S.G., 2018. Dengue and Zika: Control and Antiviral Treatment Strategies, vol 1062 Springer.
Kato, F., Hishiki, T., 2016. Dengue virus reporter replicon is a valuable tool for antiviral Drug discovery and analysis of virus replication mechanisms. Viruses 8 (5).
Li, X.D., et al., 2016. Transmembrane domains of NS2B contribute to both viral RNA replication and particle formation in Japanese encephalitis virus. J. Virol. 90 (12), 5735–5749.
Li, A., et al., 2018. A Zika virus vaccine expressing premembrane-envelope-NS1 polyprotein. Nat. Commun. 9 (1), 3067.
Lu, C.Y., et al., 2017. Single-round infectious particle antiviral screening assays for the Japanese encephalitis virus. Viruses 9 (4).
Mutso, M., et al., 2017. Reverse genetic system, genetically stable reporter viruses and packaged subgenomic replicon based on a Brazilian Zika virus isolate. J. Gen. Virol. 98 (11), 2712–2724.
Su, J., et al., 2011. Duck egg-drop syndrome caused by BYD virus, a new Tembusu-related flavivirus. PLoS One 6 (3), e18106.
Ti, J., et al., 2016. Duck Tembusu virus exhibits pathogenicity to kunming mice by intracerebral inoculation. Front. Microbiol. 7, 190.
Wang, H.J., et al., 2016. The emerging duck flavivirus is not pathogenic for primates and is highly sensitive to mammalian interferon antiviral signaling. J. Virol. 90 (14),

6538–6548.
Xie, X., et al., 2016. Zika virus replicons for Drug discovery. EBioMedicine 12, 156–160.
Yamanaka, A., Suzuki, R., Konishi, E., 2014. Evaluation of single-round infectious, chimeric dengue type 1 virus as an antigen for dengue functional antibody assays. Vaccine 32 (34), 4289–4295.
Zhang, W., et al., 2017. An updated review of avian-origin Tembusu virus: a newly emerging avian Flavivirus. J. Gen. Virol. 98 (10), 2413–2420.
Zhu, K., et al., 2015. Identification and molecular characterization of a novel duck Tembusu virus isolate from Southwest China. Arch. Virol. 160 (11), 2781–2790.