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Letter

# A noninfectious pseudovirus system for an emerging orthoflavivirus

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***Dear Editor,***

For the past few decades, mosquito-borne orthoflaviviruses, such as dengue virus, Zika virus, and West Nile virus (WNV), have posed significant threats to global public health. The *Orthoflavivirus* genus comprises more than 70 viruses, many of which are responsible for a wide range of diseases in humans and animals, including febrile illnesses, encephalitis, and hemorrhagic febrile illness. These viruses are associated with approximately 400 million infections and 100 million symptomatic cases worldwide each year (Van Leur et al., 2021). Among them, Usutu virus (USUV) is an emerging pathogen classified alongside WNV within the Japanese encephalitis virus (JEV) serogroup of the *Orthoflavivirus* genus, the *Flaviviridae* family. USUV has spread from Africa to Europe since the late 20th century. It primarily causes central nervous system disorders in birds, with several large-scale mortality events recorded in Europe. USUV can also infect humans, it typically leads to neurological complications in rare cases (Roesch et al., 2019). Humans and other mammals are considered dead-end hosts, whereas birds serve as the primary amplifying hosts (Golding et al., 2023). Given its increasing incidence in human infections and expanding geographic distribution, USUV presents an emerging public health threat.

At present, there is an urgent need for research tools to study the molecular virology of USUV. Although USUV is currently classified as a biosafety level-2 (BSL-2) pathogen (Golding et al., 2023), live virus experiments have conventionally been conducted in BSL-3 facilities due to safety concerns (Blazquez et al., 2013; Cvjetković et al., 2017). To date, only infectious clones have been established for the USUV (Bates et al., 2021). The stringent biosecurity requirements and absence of non-infectious molecular virology tools have hindered progress in USUV research. To overcome these dual challenges, orthoflavivirus pseudovirus system represent ideal molecular tool to study the viral characteristics while eliminating the risk of laboratory infection.

In this study, we attempted to generate a ΔNS1-pseudovirus system for USUV. To produce the ΔNS1-pseudovirus efficiently through *trans*-complementation, we generated a BHK-21 cell line (BHK-21-NS1) that stably expresses the USUV NS1 protein via lentiviral transfection (Fig. 1A). To prevent recombination risks in the ΔNS1-pseudovirus system, synonymous mutations were engineered into the *NS1* gene of the lentiviral expression plasmid, specifically targeting amino acid regions 1–4 and 298–352. Three monoclonal cell clones were screened by Western blotting and RT-PCR (Fig. 1B). Based on expression data and cell condition, we selected clone No.3 for subsequent experiments. To assess the stability of BHK-21-NS1 cells, the cells were passaged for ten rounds. Both Western blotting and RT-PCR analyses confirmed stable expression of the USUV NS1 protein after ten passages (Fig. 1C).

Following a strategy similar to that used for Kunjin virus (KUNV) and yellow fever virus (Lindenbach and Rice, 1997; Li et al., 2019), we constructed a truncated genome of USUV strain *Turdus merula* Netherlands 2016 (i.e., "TMN", GenBank: MN813490.1) with a 97-amino acid in-frame deletion in the *NS1* gene, generating the TMN-ΔNS1 construct. This construct retained the first 156 N-terminal and the last 99 C-terminal residues of the NS1 protein (Fig. 1D). The infectious cDNA clone for the USUV TMN strain, a gift from Professor James Weger-Lucarelli (Virginia-Maryland College of Veterinary Medicine, USA), was used as the template for plasmid construction. Next, the TMN-ΔNS1 plasmid was transfected into BHK-21-NS1 cells or naïve BHK-21 cells. Supernatants were collected at day 3 post-transfection and

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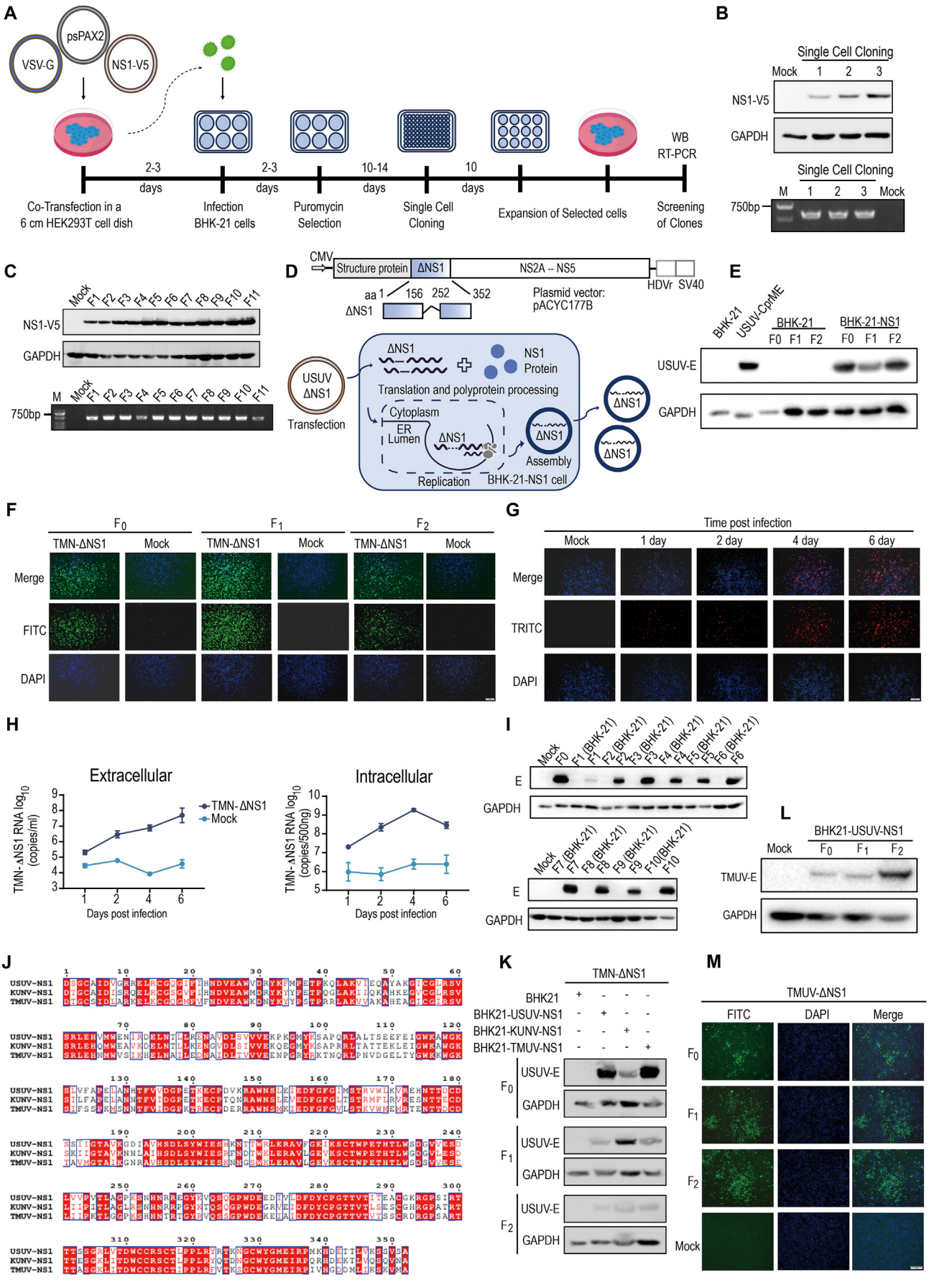


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**Fig. 1.** Establishment and characterization of the USUV-ΔNS1 pseudovirus system. **A** Schematic of BHK-21 stable cell lines expressing USUV NS1–V5 protein. **B** NS1–V5 protein expression and *NS1* gene expression in three single-cell clones were analyzed by Western blotting (WB) and RT-PCR using cell lysates harvested 48 hours post-passaging. **C** To assess the stability of NS1 expression in BHK-21-NS1 cells passaged through multiple generations, cells were cultured daily up to the F11 generation, followed by analysis of NS1 protein (WB) and gene expression (RT-PCR) using anti-V5 rabbit mAbs on lysates harvested 48 hours post-passaging. **D** Design of the TMN-ΔNS1 plasmid construct and workflow of the ΔNS1 pseudovirus system. **E** WB analysis of TMN-ΔNS1 pseudovirus E protein expression across F0–F2 generations following transfection (F0) or infection (F1 and F2) into BHK-21-NS1 or naïve BHK-21 cells. The supernatants were harvested at 3 days post-transfection, subjected to two sequential passages on respective cells, and pseudovirus production were evaluated through intracellular E protein detection. **F** IFA identification of TMN-ΔNS1 pseudovirus (F0–F2) in BHK-21-NS1 cells (experimental group) and naïve BHK-21 cells (mock control) at 72 h post-transfection or infection. E protein (green) was detected using a mouse monoclonal antibody (mAb) against USUV-E; nuclei were counterstained with DAPI (blue). Scale bar = 100 μm. **G** Time-course IFA detection of TMN-ΔNS1 pseudovirus E protein expression at 1, 2, 4, and 6 days post-infection (dpi) in BHK-21-NS1 cells or naïve BHK-21 cells(mock). E protein (red) was visualized using a rabbit mAb against USUV-E; nuclei were stained with DAPI (blue). Scale bar = 100 μm. **H** RT-qPCR quantification of intracellular versus extracellular TMN-ΔNS1 RNA levels. Data are presented as means ± standard deviation. **I** Genetic stability assessment of TMN-ΔNS1 pseudovirus. BHK-21-NS1 and naïve BHK-21 cells were infected with TMN-ΔNS1 pseudovirus (F0–F9 generations) and harvested at 72 h post-infection for analysis of viral E protein expression by WB using a rabbit monoclonal antibody (mAb) against USUV-E. **J** Multiple sequence alignment of flavivirus NS1 proteins generated using ESPript 3.0. Conserved residues are highlighted with a red background, highly conserved residues are shown in red text, and regions of high conservation are boxed in blue. **K** Efficient replication of USUV TMN-ΔNS1 pseudovirus (F0–F2 generations) in BHK-21-KUNV-NS1 and BHK-21-TMUV-NS1 cells. E protein expression was confirmed by WB analysis of cell lysates harvested at 72 h post-infection. **L, M** Confirmation by WB (L) and IFA (M) that USUV-NS1 supports TMUV-ΔNS1 pseudovirus replication following two serial passages (F0–F2) in BHK-21-USUV-NS1 cells. Analysis was performed on cells harvested at 72 h post-infection. NS3 protein (green) and DAPI-stained nuclei (blue) are shown. Scale bar = 100 μm.

passaged twice on the respective cells. Pseudovirus production was assessed by detecting intracellular levels of the envelope (E) protein. Western blotting and indirect immunofluorescence assays (IFA) revealed that positive signals were only detected in BHK-21-NS1 cells, but not in naïve BHK-21 cells (Fig. 1E and F). These results demonstrate that the TMN-ΔNS1 construct is replication-defective in naïve BHK-21 cells but can be rescued and replicated in BHK-21-NS1 cells.

To evaluate the replication efficacy of TMN-ΔNS1 through the *trans*-complementation approach, we measured its growth kinetics in BHK-21-NS1 cells. The supernatant from the F0 generation of the ΔNS1-pseudovirus was collected to infect fresh BHK-21-NS1 cells. IFA fluorescence signals accumulated over time, corresponding with an increase in viral RNA copies in both intracellular and extracellular samples (Fig. 1G and H). Intracellular viral RNA reached its peak at 4 days post-infection, while extracellular RNA reached its maximum level at 6 days post-infection, possibly indicating ongoing replication. Both intracellular and extracellular levels of viral RNA showed approximately a 100-fold increase compared to the initial levels detected at 1 day post-infection. These results confirm the efficient replication of TMN-ΔNS1 pseudovirus in BHK-21-NS1 cells.

To verify the safety of the TMN-ΔNS1 system, the pseudovirus was continuously passaged for 10 rounds in BHK-21-NS1 cells. Subsequently, the resultant TMN-ΔNS1 viruses from passages F1 to F10 were used to infect naïve BHK-21 cells to detect any replication-competent virus production through potential recombination events. As shown in Fig. 1I, no detectable viral E protein was produced after infection of naïve BHK-21 cells, even after the TMN-ΔNS1 pseudovirus underwent ten rounds of passage in BHK-21-NS1 cells. Collectively, these data confirm that a high yield of TMN-ΔNS1 virus was obtained in BHK-21-NS1 cells without any recombination into replication-competent viruses during the passages.

The NS1 protein of orthoflavivirus is a multifunctional and versatile protein involved in immune evasion, pathogenesis, and viral replication (Youn et al., 2013). The USUV-NS1 protein shares 74.7% sequence identity with Kunjin virus (KUNV)-NS1 and 63.4% identity with Tembusu virus (TMUV)-NS1, as illustrated in Fig. 1J. We examined the ability of NS1 from various orthoflaviviruses to *trans*-complement TMN-ΔNS1 in both transfection and infection assays. BHK-21 cells expressing KUNV-NS1 and TMUV-NS1 were used for this assay. Upon transfection, both KUNV-NS1 and TMUV-NS1 were able to rescue the replication of TMN-ΔNS1 (Fig. 1K). After two passages on these cells, the USUV envelope (E) protein was still clearly detected by Western blotting. These findings suggest two complementary strategies that enhance safety by reducing the likelihood of TMN-ΔNS1 reverting to a wild-type phenotype.

Next, we tested whether USUV-NS1 could support the replication of heterologous orthoflavivirus by *trans*-complementation. A cDNA clone of TMUV-ΔNS1, featuring a deletion in the *NS1* gene identical to that of TMN-ΔNS1, was transfected into BHK-21-TMN-NS1 cells. A significant number of IFA-positive cells were observed, and the expression of TMUV-NS3 protein was clearly detected by Western blotting, even after the supernatants were passaged twice in cells (Fig. 1L and M). These results demonstrate that BHK-21-TMN-NS1 successfully supported the replication of TMUV-ΔNS1. Altogether, the data suggest significant genetic and functional similarity of the NS1 protein among viruses in the *Orthoflavivirus* genus. This finding highlights NS1 is potential for developing broad-spectrum antivirals and therapeutics that target multiple orthoflaviviruses.

Currently, infectious clones have only been established for the TMN and UG09615 2010 strains of USUV (Bates et al., 2021), and the lack of available molecular tools impedes basic research into the viral biology and the development of effective antivirals. In this study, we constructed the ΔNS1-pseudovirus system. This convenient tool mimics virus infection *in vivo* and eliminates the need for handling infectious viruses. It can be used for vaccine development, viral replication studies and more. This innovative tool will undoubtedly accelerate the development of targeted antivirals and enhance our understanding of USUV replication mechanisms, advancing research into USUV biology.

## FOOTNOTES

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