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RESEARCH ARTICLE

Infectious clone of a contemporary Tembusu virus and replicons expressing reporter genes or heterologous antigens from poultry viruses

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Abstract

The novel mosquito-borne Tembusu virus (TMUV, family *Flaviviridae*) was discovered as the cause of a severe outbreak of egg-drop syndrome affecting ducks in South-east Asia in 2010. TMUV infection can also lead to high mortality in various additional avian species such as geese, pigeons, and chickens. This study describes the construction of an infectious cDNA clone of a contemporary duck-isolate (TMUV WU2016). The virus recovered after transfection of BHK-21 cells shows enhanced virus replication compared to the mosquito-derived MM1775 strain. Next, the WU2016 cDNA clone was modified to create a SP6 promoter-driven, self-amplifying mRNA (replicon) capable of expressing a range of different reporter genes (Renilla luciferase, mScarlet, mCherry, and GFP) and viral (glyco)proteins of avian influenza virus (AIV; family *Orthomyxoviridae*), infectious bursal disease virus (IDBV; family *Bunyaviridae*) and infectious bronchitis virus (IBV; family *Coronaviridae*). The current study demonstrates the flexibility of the TMUV replicon system, to produce different heterologous proteins over an extended period of time and its potential use as a platform technology for novel poultry vaccines.

KEYWORDS
duck Tembusu virus, infectious clone, platform technology, replicon, self-amplifying mRNA, vaccine, viral poultry diseases

1 | INTRODUCTION

Recombinant viral vectors are very powerful tools for gene delivery into eukaryotic cells. These vectors are often applied as human or veterinary vaccine platforms and used in the gene therapy field. The different strategies developed over the years have mostly focused on plasmid-based expression vectors, or large dsDNA viruses such as adeno-, pox- or herpesviruses. More recently, positive-stranded RNA viruses also gained interest for the application as heterologous gene expression platforms.[1–3] The RNA of positive-stranded RNA viruses serves as a messenger RNA (mRNA) that can be directly translated in transfected cells. Several members of the *Togaviridae* family such as Venezuelan equine encephalitis virus (VEEV) and Semliki Forest virus (SFV) and of the *Flaviviridae* family such as West Nile virus - strain Kunjin (KUNV) have been engineered as efficient viral 'replicon' vectors for high-level transgene expression for applications in gene therapy, recombinant protein production or as vaccine platform technology against emerging viral diseases.[4–8] By removing the structural

and maintaining the nonstructural genes, efficient RNA replication and translation from the replicon is achieved without the formation of infectious viral progeny.

Tembusu virus (TMUV) is an emerging epornitic flavivirus that was initially isolated from *Culex tritaeniorhynchus* vector mosquitoes in 1955 in Malaysia.[9] TMUV remained obscure until the major outbreak of the disease in ducks in 2010 in China,[10] which had a significant economic impact on the poultry industry. Since then, TMUV has been widely detected in other avian species such as chickens, pigeons, and sparrows.[11] Some studies have reported TMUV-specific antibodies in humans,[12,13] but flavivirus serodiagnosis is complicated due to extensive cross-reactivity with other species within the *Flavivirus* genus.[14,15] Experimental infections suggest that TMUV is highly sensitive to interferon and cannot replicate well in primates.[16]

The TMUV genome is approximately 11 kb in size and encodes a single polyprotein that is post-translationally processed into the three structural proteins capsid (C), premembrane (prM), and envelope (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) involved in viral replication and modulation of host responses. The non-structural proteins NS3 (helicase) and NS5 (RNA-dependent RNA polymerase) are part of the replication complex responsible for the synthesis of the double-stranded (ds)RNA replication intermediate and positive-sense viral progeny RNA. Other functions of the nonstructural proteins include the proteolytical processing of the polyprotein by the protease NS2B-NS3, capping of the viral RNA genome by the methyltransferase activity of NS5, and interfering with the host interferon pathway.[17–19] The structural proteins C, prM, and E are involved in the packaging of the viral RNA genome into virions. Although many of the TMUV virus-encoded proteins functions have been studied to various extents,[20,21] the use of the TMUV replicon system for the expression of heterologous virus antigens has not been described.

To obtain a better understanding of the replication process of TMUV and investigate the possibilities for transgene expression, this paper shows a reverse genetic approach to generate a full-length cDNA clone and a series of SP6 promoter-driven non-infectious subgenomic replicons of the contemporary TMUV isolate WU2016. Furthermore, this study demonstrates the long-lasting expression of reporter genes and the production of poultry-specific viral antigens by the TMUV replicon in BHK-21 cells.

2 | MATERIAL AND METHODS

2.1 | Construction of the TMUV WU2016 infectious cDNA clone

Viral RNA was extracted by using TRIzol reagent (Invitrogen, catalog number (*CN*):*15596026*) and reverse transcribed by SuperScript II (Invitrogen) using random hexanucleotides. PCR primers were designed to create overlapping subgenomic fragments (F1 to F6, Figure 1A) that were subsequently cloned into pJET1.2 and sequenced by Sanger sequencing (Macrogen). Primer sequences were based on the closely related DF-2 DTMUV isolate (GenBank: KJ489355.1) (Table 1). The individual fragments were designed to allow for the assembly of the TMUV WU2016 cDNA clone using restriction cloning. F1 was extended by inserting an SP6 promoter sequence upstream of the 5′ untranslated region (UTR) to allow for in vitro RNA transcription of the viral genome. F6 was extended by including a hepatitis delta virus (HDV) ribozyme after the last nt of the 3′UTR to ensure a native 3′ terminus and the *PacI* restriction site (primer 13 & 14, Table 1). The copy-control pCC1BAC vector (Epicentre Biotechnologies, *CN: V008675*)[22] was used for the assembly of the individual fragments via ligation cloning with *EcoRI* and *AatII* (Fragment 1), *AatII* and *NheI* (fragment 2), *NheI* and *BbvCI* (fragment 3), *BbvCI* and *NsiI* (Fragment 4), *NsiI* and *BamHI* (fragment 5) and *BamHI* and *PacI* (fragment 6). The TMUV WU2016 infectious cDNA clone or intermediates were transformed into copy-control EPI300 *E. coli* (Lucigen, *CN:C300C105*) and induced using CopyControl Solution (Lucigen, *CN:C300C105*). DNA was isolated using the Nucleobond Xtra Midi Kit (Macherey-Nagel, *CN: MN740412*) and checked for quantity and quality using the spectrophotometer ND-1000 (Nanodrop). The complete sequence was confirmed by Sanger sequencing of intermediate clones and by Oxford Nanopore sequencing of the entire cDNA clone (Plasmidsaurus) and submitted to GenBank (accession no. OQ920272).

2.2 | Construction of TMUV subgenomic replicons expressing reporter genes and heterologous poultry virus antigens

A synthetic DNA fragment was designed containing the SP6 promoter preceding the 5′UTR and the first 60 nt of the coding region of the capsid (C) gene required for translation initiation and cyclization of the viral genome.[23] The last 22 amino acids of the envelope (E) protein, non-structural gene cassette, 3′UTR, and HDVr were amplified using PCR (primer 14 & 15, Table 1) from the TMUV WU2016 cDNA and ligated to the synthetic DNA fragment (Figure 1A). The unique restriction sites *AscI* and *AvrII* were added for insertion of the reporter genes; GFP, mScarlet, mCherry, and Renilla luciferase (Rluc) into the subgenomic replicon. The reporter genes were inserted between the remaining capsid gene and the retained 66 nt of the E gene that encodes the signal sequence to ensure translocation into the endoplasmic reticulum of the downstream NS1 protein. The reporter genes were followed by the sequence for the ribosomal skipping element 2A of the foot-and-mouth disease virus (FMDV). The construct TMUVrep-HA was obtained by inserting the codon-optimized hemagglutinin (HA) (high pathogenic avian influenza A virus (AIV; *Orthomyxoviridae* family) H5N1 A/turkey/Turkey/01/05) gene isolated from an expression plasmid kindly provided by MSD Animal Health. For the TMUVrep-Spike construct, the spike (S) protein of infectious bronchitis virus (IBV; *Coronaviridae* family) strain Ma5 (Serotype Massachusetts) was obtained as a synthetic gene fragment (GeneArt, Thermo Fisher Scientific). For the TMUVrep-pVP2 construct, the precursor VP2 (pVP2) gene of infectious bursal disease virus (IBDV; *Birnaviridae* family) strain Faragher 52/70 was isolated using high-fidelity PCR amplification with Q5 High-

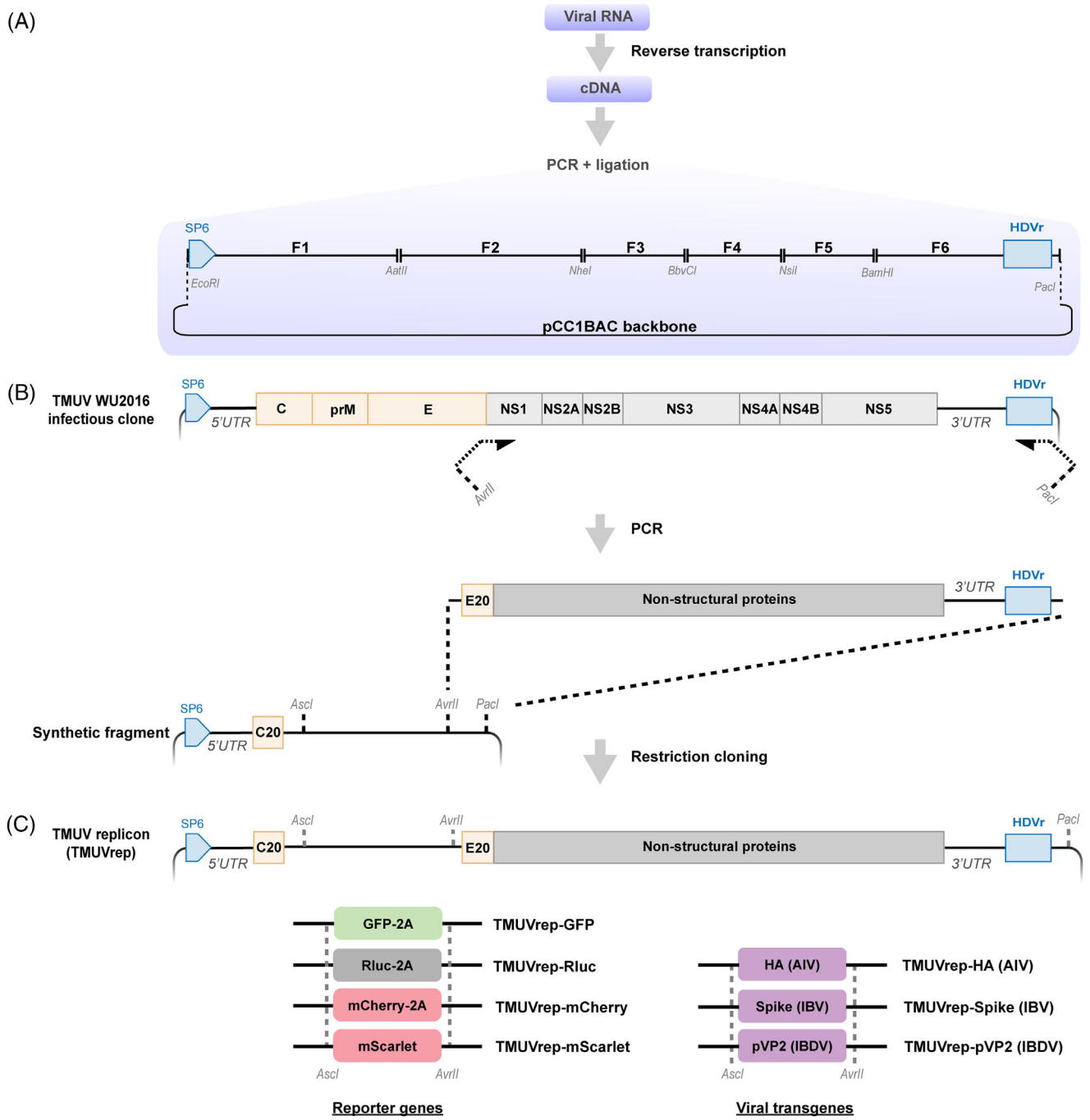

FIGURE 1 Schematic representation of the TMUV genome, TMUV WU2016 infectious cDNA clone, and TMUV replicon. (A) Purified TMUV WU2016 RNA was used as a template in a reverse transcriptase reaction for the generation of cDNA. Next, the cDNA was amplified into 6 fragments (F1 to F6) using TMUV-specific primers and ligated into a SP6-containing pCC1BAC backbone creating the (B) TMUV WU2016 infectious cDNA clone. The TMUV genome consists of a single open reading frame flanked by 5′ and 3′ untranslated regions (UTR). The genome translates into a single polyprotein consisting of three structural proteins capsid (C), premembrane (prM), envelope (E) protein, and five non-structural proteins (NS1-5). The TMUV WU2016 infectious cDNA clone served as a template for the construction of the (C) subgenomic replicon RNA by PCR amplifying the last 22 amino acids of E protein, non-structural proteins, 3′UTR, and hepatitis delta virus ribozyme (HDVr) and ligating it into a synthetic fragment encoding the SP6, 5′UTR and first 20 amino acids of the C protein. Lastly, the TMUV replicon was modified via AscI and AvrII restriction digestion to express the green fluorescent protein (GFP), Renilla luciferase (Rluc), or mCherry flanked by foot-and-mouth disease virus 2A elements or mScarlet reporter proteins. For the expression of viral transgenes, the hemagglutinin (HA) of avian influenza A virus (AIV), Spike of infectious bronchitis virus (IBV), or the nucleocapsid (pVP2) gene of infectious bursal disease virus (IBDV) was inserted into the TMUV replicon.

TABLE 1 List and sequence of primers used for the TMUV WU2016 infectious clone and replicon construction.

No.	Fragment	Sequence (5′ – 3′)
1	SP6-F1-fw	atttaggtgacactatagagaagttcatctgtgtgaact
2	F1-rv	agcataagttgccttggg
3	F2-fw	tcgaccaaagccactaaatatc
4	F2-rv	tgctgctgtcatcaaactg
5	F3-fw	ctgagagccgtgtttgaag
6	F3-rv	tccgactatctatgacccg
7	F4-fw	gacaaagaaggacaggtg
8	F4-rv	cctcaaggtctggaacatct
9	F5-fw	agctacaacatttctgactcc
10	F5-rv	atggctgacaacctgttc
11	F6-fw	atcgtggcaagatggatg
12	F6-rv	agactctgtgttctaccacc
13	F6-ext-fw[a]	caagctgtaactctaggggaa
14	F6-ext-rv[a]	agaaagatgcggccctaattaaac
15	E22-fw	ttggcgcgccaacgcctaggctccatttctatgacttttctagcc

[a]Used to extend the F6 amplicon with HDVr element and *PacI* site.

Fidelity DNA Polymerase (NEB, *CN: M0491L*) from a template provided by MSD Animal Health (Boxmeer) and sequence verified using sanger sequencing (Macrogen). All the viral antigens were inserted into the compatible *AscI/AvrII* restriction sites of the TMUVrep construct.

2.3 | In vitro RNA transcription and RNA purification

Plasmids were purified using the endotoxin-free Nucleobond Midiprep kit (Macherey-Nagel, *CN: MN740412*) and linearized using *PacI*. Capped, in vitro transcribed viral or replicon RNA was synthesized using SP6 polymerase (New England Biolabs, *CN: M0207S*) and 500 ng of the unpurified linearized plasmid DNA in a total volume of 40 μL. The total reaction mixture was incubated for 2 h at 37°C and afterward treated for 30 min at 37°C with RNAse-free DNAse (Promega, *CN: M6101*). The RNA was purified using the RNeasy Micro kit (Qiagen, *CN: 74004*) according to the manufacturer's instructions. Both the quantity and quality of the in vitro transcribed RNA were analyzed using the spectrophotometer ND-1000 (Nanodrop) and via conventional electrophoresis using a 1% agarose gel in tris-acetate-EDTA (TAE) buffer for 15 min at 150 V.

2.4 | Cells and virus preparation

Baby hamster kidney cells (BHK-21; clone 13, ECACC 85011433) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, *CN: 41966052*) supplemented with 10% fetal bovine serum (FBS; Gibco, *CN:10270106*) and 100 U mL^{-1} penicillin-streptomycin (Gibco, *CN: 15140122*). Cells were cultured at 37°C under 5% CO_2. A subconfluent 6-well plate containing BHK-21 cells was transfected with approximately 10 micrograms in vitro transcribed RNA of the TMUV WU2016 infectious clone using Lipofectamine 2000 (Invitrogen, *CN: 11668019*). The supernatant of the transfected cells was used to infect healthy BHK-21 cells for the generation of the passage 1 (P1) virus stock. P5 virus stocks of TMUV MM1775 (Genbank accession no. JX477685; EVAg Ref-SKU 001v-EVA135) were propagated on BHK-21 cells. The viral titer was determined on chicken embryonic fibroblast (DF-1) cells using the end-point dilution assay (EPDA) and expressed as median tissue culture infectious dose ($TCID_{50}$)/mL according to the Reed–Muench method. Virus samples were 10-fold serially diluted, mixed with DF-1 cells, and distributed on 60-well microtiter plates. The plates were incubated for 5–7 days. The scoring of the plates was done by the observation of cytopathic effect (CPE) using brightfield microscopy.

2.5 | Cell electroporation

Electroporation of TMUV replicon RNA into BHK-21 cells was performed using the Gene Pulser Xcell (Bio-Rad Laboratories). A total of 8×10^6 BHK-21 cells were harvested and resuspended in 1 mL Dulbecco's phosphate-buffered saline (DPBS; Gibco, *CN: 14190144*). Next, 10ug of purified replicon RNA was added to the resuspended BHK-21 cells and transferred to a 0.4 cm cuvette (Bio-rad Laboratories, *CN: 1652088*). Subsequently, the cuvette was pulsed twice (850 V/25 μF) and cells were resuspended in 10 mL supplemented DMEM. Hereafter, cells were processed in plates according to the application and incubated at 37°C under 5% CO_2.

2.6 | Viral growth kinetics

The viral growth kinetics were studied in BHK-21 cells. Cells were seeded in a 6-well plate with a concentration of 1×10^6 cells/mL and inoculated at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$ per cell. After 2 h of incubation, the supernatant was removed and the cells were washed once with DPBS. DMEM+HEPES (Gibco, *CN: 10564011)* supplemented with 10% FBS (Gibco, *CN:10270106*) and 100 U mL^{-1} penicillin-streptomycin (Gibco, *CN: 15140122*) was added to the infected cells and incubated at 37°C under 5% CO_2. The supernatants were collected every 24 h for 5 days and stored at −80°C prior to titration by EPDA on DF-1 cells.

2.7 | Indirect immunofluorescence assay

An indirect immunofluorescence assay (IFA) was performed to detect the presence of viral proteins or viral double-stranded RNA (dsRNA) in infected or replicon-transformed cells. A monolayer of BHK-21 cells was washed with DPBS and fixed with 4% paraformaldehyde (Thermo Fisher Scientific, *CN: J61899.AP*) in DPBS at RT for 5 min. The cells

were washed and permeabilized using 0.1% sodium dodecyl sulfate (SDS) in DPBS at RT for 10 min. Next, the monolayer was blocked by DBPS supplemented with 5% FBS at 37°C for 1 h and incubated at 37°C for 1 h with primary antibodies pan-flavivirus α-NS1 (1:50, 4G4; mouse), pan-flavivirus α-E (1:100, 4G2; mouse), α-dsRNA (1:32, 3G1.1; mouse), serum α-HA (1:500, strain H5N1; chicken), α-VP2 (1:500, R63; mouse),[24] or α-S (1:50, strain M41 IBV; mouse) diluted in DPBS containing 5% FBS. Hereafter, cells were incubated at 37°C for 1 h with a secondary α-mouse IgG conjugated with Alexa Fluor-488 (goat; 1:2000; Invitrogen, *CN: A32723*), Alexa Fluor-546 (goat, 1:2000; Invitrogen, *CN A11030*), or α-chicken IgY conjugated with Alexa Fluor-488 (goat; 1:2000; Invitrogen, *CN: A11039*) antibody in DPBS containing 5% FBS. Cells were then stained with Hoechst (1:100; Thermo Fisher Scientific, *CN: 11594876*) in DPBS for 5 min. Images were acquired using an inverted fluorescence microscope (Axio observer Z1; Zeiss).

3 | RESULTS

3.1 | Construction of the TMUV WU2016 infectious clone and sub-genomic replicons

To create an infectious cDNA clone of duck Tembusu virus isolate WU2016, the RNA was isolated and used for RT-PCR amplification (Figure 1A). The six overlapping PCR fragments were cloned, sequenced, and then assembled in a single-copy pCC1BAC backbone by restriction cloning (Figure 1B). The subgenomic replicon (TMUVrep) was constructed by deleting part of the coding sequence of the structural genes from the TMUV WU2016 cDNA, retaining only the first 60 nucleotides of the capsid coding sequence (C_{20}) and the last 66 nucleotides of the envelope protein coding sequence (E_{22}). C20 is necessary to initiate the translation of the polyprotein and for cyclization of the replicon RNA,[25] whereas E22 encodes the signal sequence required for proper translocation of the nonstructural protein 1 (NS1) into the endoplasmic reticulum (ER).[23] To study the function of the constructs to replicate and express heterologous proteins, the replicon was subsequently modified by cloning the reporter genes (e.g., GFP, Rluc, mScarlet and mCherry) or viral transgenes HA, (AIV) Spike (IBV) or pVP2 (IBDV) in frame with the ORF (Figure 1C).

3.2 | Characterization of the TMUV WU2016 infectious clone

An infectious virus was generated by transfection of capped, in vitro transcribed TMUV WU2016 RNA into BHK-21 cells. In order to analyze the growth kinetics of TMUV WU2016 and compare this to the prototypical TMUV MM1775 isolate, BHK-21 cells were infected at an MOI of 0.01 $TCID_{50}$/mL and samples were taken every day for the duration of 4 days (Figure 2A). The highest titer was detected at 72 hpi (1.33×10^8 $TCID_{50}$/mL) and 48 hpi (6.08×10^6 $TCID_{50}$/mL) for TMUV WU2016 and MM1775, respectively. Clear cytopathic effect in BHK-21 cells was observed at 96 h post-infection (hpi) (Figure 2B). Comparative sequence analysis of TMUV WU2016 showed an 89.1% nucleotide sequence and 97.0% protein sequence homology to the TMUV MM1775 isolate. The TMUV WU2016 most closely relates to the DTMUV DF-2 isolate with 99.9% nucleotide and 99.9% protein homology (Figure 2C).

3.3 | Characterization of the TMUV replicon

To verify the functionality of the TMUV replicon, capped in vitro transcribed RNA was produced from the TMUVrep-GFP cDNA and electroporated in BHK-21 cells, which were analyzed using (immuno)fluorescence microscopy 48 h post-electroporation (hpe). As a control, BHK-21 cells were infected with TMUV WU2016 at an MOI of 0.01. The TMUV E and NS1 proteins were detected using monoclonal antibodies (mAb) 4G2 and 4G4, respectively. Additionally, the presence of double-stranded RNA replication intermediates within the flavivirus RNA replication complex was detected using mAb 3G1.1. NS1 and dsRNA are detected in cells electroporated with TMUVrep-GFP or infected with TMUV WU2016 (Figure 3). NS1 was detected in the cytoplasm, where a perinuclear localization was observed. Similarly, dsRNA intermediates were detected in the perinuclear region for both the TMUV replicon and the TMUV WU2016 infectious clone. As expected, the TMUV envelope protein could only be detected in the samples infected with the TMUV WU2016 infectious clone, whereas GFP expression could only be detected in cells electroporated with TMUVrep-GFP.

In order to analyze the expression and timing of reporter protein expression, capped, in vitro transcribed RNA of TMUVrep-GFP, TMUVrep-mScarlet, TMUVrep-mCherry, or TMUVrep-Rluc was electroporated into BHK-21 cells (Figure 4A). Transgene expression was measured over four consecutive days. The fluorescent reporter proteins (GFP, mScarlet) were analyzed using fluorescence microscopy and showed the highest fluorescence expression between 48 and 72 h post-electroporation (hpe). This was also confirmed for TMUVrep-eGFP-transformed cells using flow cytometry analysis (Figure 4B). Moreover, a strictly cytoplasmic localization of mScarlet was observed in the absence of the FMDV-2A site while this is not seen for GFP and mCherry, both of which are dispersedly distributed in the entire cell (Figure 4C). The Rluc activity was detected from cellular lysate prepared from BHK-21 cells and peaked at 48 hpe (Figure 4D).

Next, the TMUV replicons encoding the HA (AIV), Spike (IBV), and pVP2 (IBDV) genes were tested for the expression and localization of the viral (glyco)proteins. The signal sequence encoded by the two glycoproteins directs both HA or Spike glycoproteins into the ER for transport to the plasma membrane. In contrast, the capsid protein pVP2 has a cytosolic localization therefore it is separated from the viral polyprotein by incorporation of FMDV-2A sites (Figure 5A).

To confirm the expression and processing of the viral (glyco)proteins, capped, in vitro transcribed replicon RNA was electroporated in BHK-21 cells, and cells were analyzed 72 hpe using an indirect immunofluorescence assay. Successful expression was detected for all TMUV replicon-expressed (glyco)proteins. Both

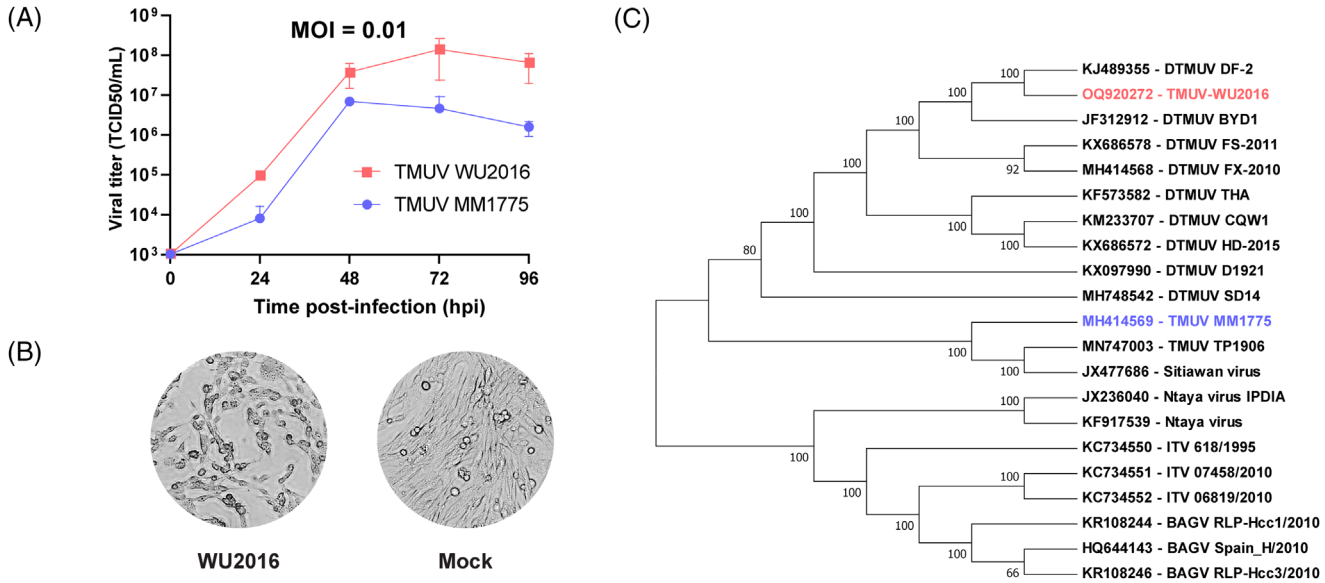

FIGURE 2 Viral growth kinetics of TMUV WU2016 compared to the TMUV MM1775 isolate. (A) BHK-21 cells were infected with TMUV WU2016 or TMUV MM1775 isolate at a multiplicity of infection (MOI) of 0.01 median tissue culture infective dose ($TCID_{50}$)/mL. Samples were taken every 24 h for up to 4 days and virus titers were determined by end-point dilution assay and expressed in $TCID_{50}$/mL. Error bars indicate the standard error of the mean titers of three biological replicates. (B) Virus-induced cytopathic effect TMUV WU2016 compared to mock-infected BHK-21 cells at 48 h post-infection (hpi). (C) Phylogeny of TMUV WU2016 (red) and TMUV MM1773 (blue) compared to other flaviviruses belonging to the Ntaya serocomplex. The phylogenetic tree was constructed using the maximum likelihood method based on the general time reversible model (GTR+G+I) of the complete genome sequences. Evolutionary analyses were conducted in MEGA7 software. The reliability of the analysis was calculated using 100 bootstrap replications. The reference nucleotide sequences of the corresponding viruses were obtained from the GenBank database.

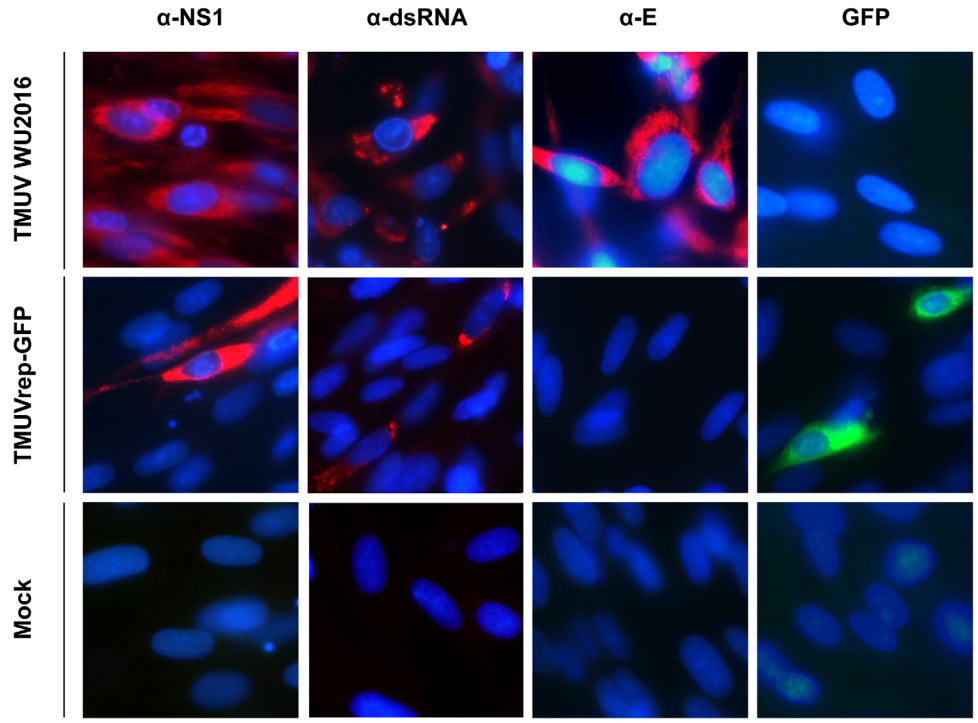

FIGURE 3 Cellular localization of viral structural, non-structural, or reporter proteins in TMUV WU2016-infected or TMUVrep-GFP-transfected BHK-21 cells. (A) An indirect immunofluorescence assay (IFA) was performed on BHK-21 cells 48 h post-infection with TMUV WU2016 (MOI 0.1 median tissue culture infectious dose ($TCID_{50}$)/mL) or post-electroporation with capped, in vitro transcribed TMUVrep-GFP replicon RNA, detecting the production of TMUV envelope (E) glycoprotein, non-structural protein 1 (NS1), or double-stranded RNA (dsRNA) replication intermediate. Primary antibodies: α-E (4G2; mouse), α-NS1 (4G4; mouse), or α-dsRNA (3G1.1; mouse) Secondary antibody: Alexa Fluor-546 conjugated α-mouse IgG (goat). Nuclei were visualized using Hoechst staining.

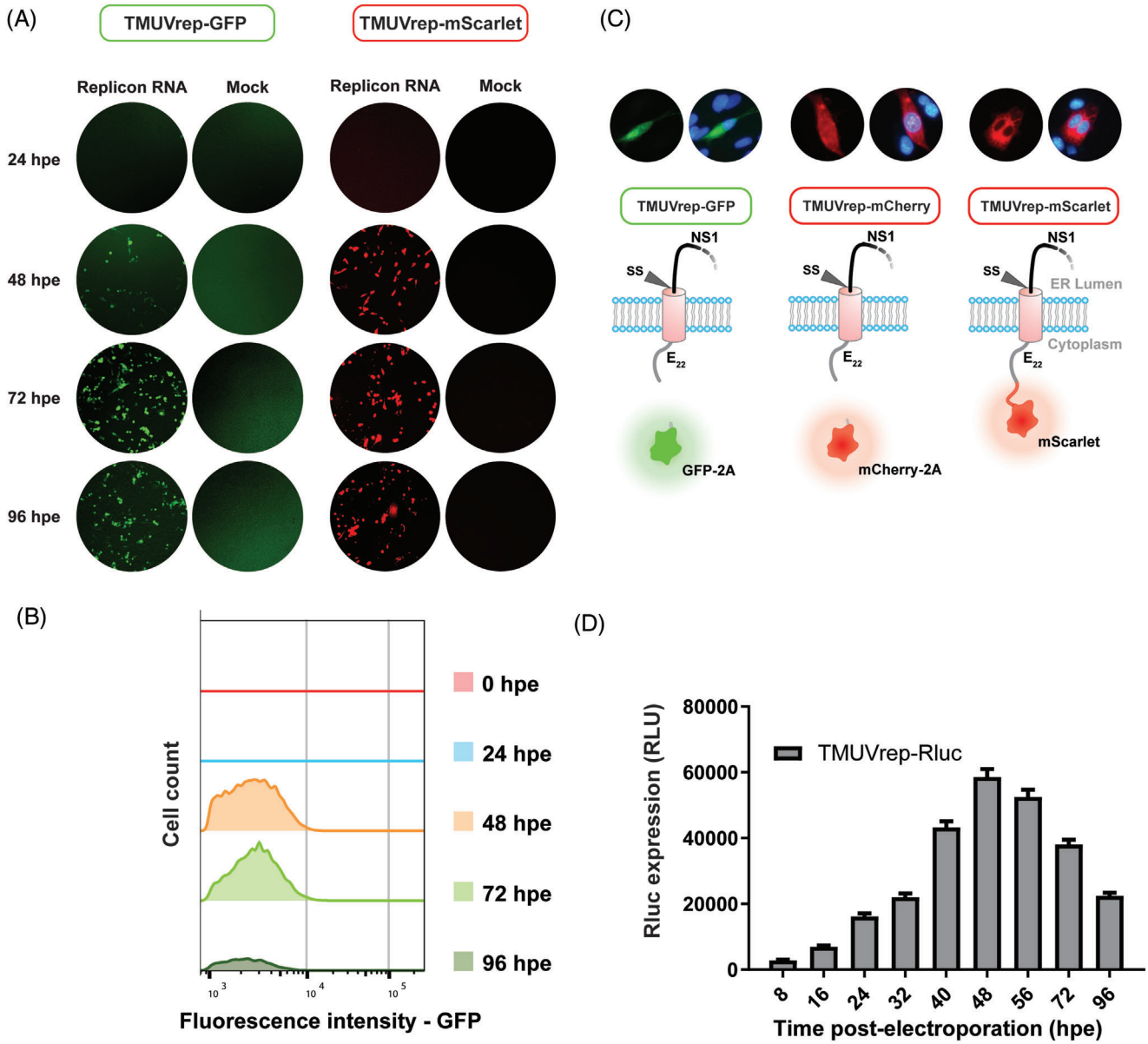

FIGURE 4 TMUV replicon-driven reporter gene expression in BHK-21 cells over time. The GFP and mScarlet expression from BHK-21 cells electroporated with capped, in vitro transcribed replicon RNA (TMUVrep-GFP or TMUVrep-mScarlet) or DPBS (mock) was determined using (A) fluorescence microscopy or, in case of TMUVrep-GFP, by (B) flow cytometry at 0, 24, 48, 72, and 96 h post electroporation (hpe). (C) The cellular localization of the fluorescent reporter proteins GFP (TMUVrep-GFP), mCherry (TMUVrep-mCherry), and mScarlet (TMUVrep-mScarlet), and the effect of the foot-and-mouth disease virus 2A element were displayed in BHK-21 cells after 48 hpe. (D) Additionally, the expression of Renilla luciferase (Rluc) from capped, in vitro transcribed replicon RNA (TMUVrep-Rluc) was analyzed using luminometry up to 96 hpe. The luminescence of Rluc was expressed in relative light units (RLU) and was measured from two independent electroporation, normalized against mock electroporated cells. The data was plotted as mean ± SEM.

glycoproteins Spike and HA showed a perinuclear localization while a more dispersed signal for the cytosolic pVP2 was detected (Figure 5B).

4 | DISCUSSION

In this manuscript, we constructed and characterized a full-length infectious cDNA clone and replicons based on the TMUV WU2016 isolate as a valuable tool to express high levels of heterologous (viral) proteins. The construction of flavivirus cDNA clones and replicons often results in genetic instability and toxicity during plasmid propagation within bacterial hosts as was described for WNV,[26] ZIKV,[27] JEV,[26] and DENV.[28] Although current prediction tools can point toward cryptic prokaryotic sites in the flavivirus genome, strategies for counteracting the toxicity resulted in highly variable outcomes.[28] It is known that not only the common viral elements such as the cytomegalovirus (CMV) promoter but also flavivirus-specific elements (e.g., the flavivirus E coding sequence) carry cryptic promoters toxic to

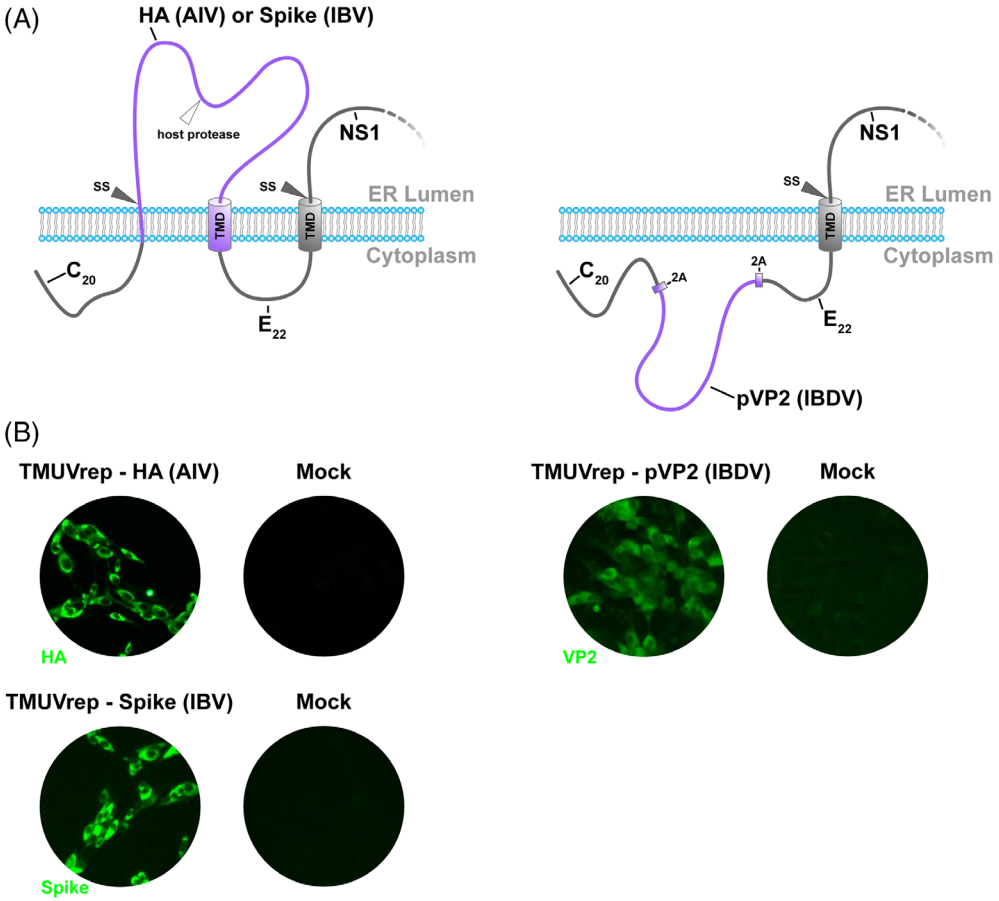

FIGURE 5 The expression of viral (glyco)proteins using the TMUV replicon in BHK-21 cells. (A) Predicted membrane topology from the polyprotein expression of TMUVrep-HA, TMUVrep-Spike shows a luminal (ER) localization, while TMUVrep-pVP2 displays a cytoplasmic localization. C20: first 20 amino acids of the capsid protein; SS: signal sequence; TMD: trans-membrane domain; E22: last 22 amino acids of the envelope (E) protein; NS1: non-structural protein 1; 2A: foot-and-mouth disease virus 2A element. (B) Detection of hemagglutinin (HA; AIV), Spike (S; IBV), and nucleocapsid (pVP2; IBDV) proteins by indirect immunofluorescence assay in BHK-21 cells electroporated with TMUVrep-HA, TMUVrep-Spike, or TMUVrep-pVP2, respectively. Primary antibodies: α-HA (chicken), α-Spike (mouse), and α-VP2 (mouse). Secondary antibodies: Alexa Fluor-488-conjugated α-chicken IgY (goat) or α-mouse IgG (goat).

bacteria.[29] To overcome genetic instability, applying a yeast cloning system or inserting an intron within cryptic promoter sites could reduce the toxic viral byproduct.[30] Although these introns do not impact the flavivirus protein coding sequence, introns can, however, disrupt higher-order RNA structures vital to efficient flavivirus replication. We circumvented problems with genetic instability by cloning the TMUV WU2016 cDNA in an inducible single-copy bacterial artificial chromosome (BAC) backbone.[31] The pCC1BAC vector is a so-called copy control plasmid typically maintained in the *E. coli* DH10β strain as a single-copy plasmid, but larger amounts of plasmid DNA can be obtained by *L-arabinose* induction of the *E. coli* strain EPI300. Indeed, no large genomic deletion or rearrangements were detected in the TMUV WU2016 cDNA when maintained in the *E. coli* DH10β strain, and the infectious virus could be successfully recovered upon transfection of capped, in vitro transcribed RNA. Future strategies could include permutating the flavivirus genome and preserving any conserved secondary and tertiary RNA motifs while reducing or completely abrogating the transcriptional activity of the cryptic bacterial promoter without affecting viral replication.[32]

Furthermore, the infectious virus was successfully recovered from BHK-21 cells transfected with in vitro transcribed RNA. The viral growth kinetics of duck-derived TMUV WU2016 were compared to that of mosquito-derived TMUV MM1775 in BHK-21 cells. TMUV WU2016 replicated faster and achieved higher overall viral titers than MM1775. The peak titers of TMUV MM1775 at 48 and 72 hpi were ~5 to 30-fold lower than those of TMUV WU2016. It was previously reported that another duck-derived TMUV strain CWQ1 led to higher peak titers than the mosquito-derived MM1775 isolate. The study described that favorable replication of the MM1775 strain in mosquito cells was most likely caused by a variable stem-loop structure in the 3′UTR of the viral genome.[33] However, similar to CWQ1, WU2016 shared high levels of sequence homology of the 3′ UTR with the CQW1 strains (97.59%) compared to the MM1775 strain (92.59%) thereby likely explaining the enhanced growth kinetics compared to MM1775.

To study the TMUV infectious cDNA clone as the basis for a potential vaccine platform technology, TMUV replicons were successfully engineered to express different reporter genes and viral transgenes. We tested the transgene expression of the WU2016-based TMUV replicon and showed a long-lasting protein production of up to 96 hpe in BHK-21 cells, without considerable cytopathic effect. It was demonstrated that the transgene expression peaked at 48 hpe confirming the results of an earlier study.[20] The apparent discrepancy between the peak expression between fluorescence reporter genes (i.e., GFP and mScarlet) and bioluminescent reporter genes (i.e., Rluc) could be explained by the reporter protein half-lives since luminescence assays are a more precise tool for measuring dynamic changes in expression levels.[34] Moreover, the presence of the FMDV-2A sites did not impair reporter protein function since both GFP and mCherry expression was detected. However, the absence of the FMDV-2A element restricts the reporter protein dispersion to the cytoplasm as was seen for mScarlet. Additionally, we demonstrated that the TMUV replicon was capable of expressing viral (glyco)proteins of AIV (HA), IBV (Spike), and IBDV (pVP2). HA was detected in the perinuclear region within electroporated cells, indicating that this viral glycoprotein was correctly routed to the cell surface as previously described for a reporter Influenza A virus.[35] Indeed, HA was also detected at the cell surface. For the TMUV replicon expressing pVP2, a cytosolic distribution was observed which is in agreement with the literature. It has been described that during the individual expression of either precursor or mature VP2 protein, the signal is located in the cytoplasm of insect, avian, and mammalian cells while in mammalian and avian cells aggregates of mature VP2 can be detected.[36]

In conclusion, we have successfully created an infectious clone and replicon system based on the TMUV WU2016 isolate that efficiently replicates in cell culture and expresses transgenes, including the viral (glyco)proteins of key poultry diseases such as AIV, IBV, and IBDV. Our results suggest that this platform may have the potential to be used in vaccination against these poultry diseases. As we continue to characterize this system, we will explore its efficacy in vivo using various delivery methods. Additionally, our work builds on prior research towards replicon RNA vaccines, which underscores the importance of developing effective and readily adaptable vaccines to combat emerging and re-emerging diseases.[7,8,37,38]

AUTHOR CONTRIBUTIONS

J.C. and G.P. designed the study. J.C., M.P., L.O., C.G., K.D., and S.C. performed the experiments. J.C. analyzed the data, wrote the manuscript draft, and prepared the figures. G.P. acquired funding, supervised the study, and edited the final version.

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CONFLICT OF INTEREST STATEMENT

The author declares no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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