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A simple method for developing an infectious cDNA clone of Japanese encephalitis virus

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Abstract Flavivirus cDNA clones frequently demonstrate genetic instability in transformed bacteria, which hampers the construction and manipulation of cDNAs for infectious flaviviruses. In this study, we developed a stable, full-length cDNA clone, pJEHEN, of a GI JEV strain HEN0701 using a medium-copy-number pBR322 vector and propagating cDNA clones at room temperature. The virus vJE-HEN recovered from the infectious clone was indistinguishable from the parent virus HEN0701 with respect to plaque morphology, growth kinetics, and virulence characteristics. A T-to-A silent mutation of nucleotide 24 of the NS2a gene was introduced into the infectious cDNA clone to eliminate frameshifting. The rescued mutant virus vJETA did not express NS1′ in infected cells and showed reduced growth and neurovirulence in mice. This convenient method for the construction and manipulation of infectious JEV cDNA clones may be of use in further studies to improve our understanding of the molecular mechanisms responsible for JEV replication and pathogenesis.

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Introduction

Japanese encephalitis is the main form of viral encephalitis in Eastern, South-eastern, and Southern Asia, with an incidence of more than 60,000 human cases per year, among which about 20–30 % of cases are fatal and up to 50 % of survivors may develop long-term neurological sequelae [1, 2]. Its causative agent, Japanese encephalitis virus (JEV), is maintained in an enzootic cycle between *Culex tritaeniorhynchus* mosquitoes and amplifying vertebrate hosts, such as water birds and domestic swine [3].

JEV is a small enveloped virus belonging to the genus Flavivirus, which includes dengue virus (DENV), west Nile virus (WNV), yellow fever virus, and tick-borne encephalitis virus, and together with WNV, Murray Valley encephalitis virus, and St. Louis encephalitis virus, it constitutes the JEV serogroup of this genus [4]. The JEV genome, like that of other flaviviruses, is a single-stranded, positive-sense RNA about 11 kb in length, comprising a single open reading frame flanked by 5′- and 3′-untranslated regions of approximately 100 and 600 nucleotides, respectively. The genomic RNA encodes a single, long polyprotein that is processed co- and post-translationally into structural (C, prM/M, E) and nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins by cellular and viral proteases [5]. The structural proteins provide the structural elements of the viral particles, while the nonstructural proteins play an essential role in viral replication and evasion of host antiviral immune responses. In addition to the ten viral proteins, a larger NS1-related protein, NS1′, is produced in cells infected with JEV [6].

Bioinformatic analysis of the genome of the JEV serogroup found a conserved canonical frameshift-stimulating motif consisting of a slippery heptanucleotide and an adjacent 38-nucleotide potential pseudoknot at the 5′ terminus of the NS2A gene [7]. It was predicted that the NS1′ protein was produced as the result of a −1 ribosomal frameshift at the slippery heptanucleotide motif. NS1′ production was subsequently shown to depend on the conserved frameshift-stimulating motifs in WNV, and NS1′ was shown to play a role in viral neurovirulence [8]. JEV also generates NS1′ in the same way as WNV, and the new −1 reading frame encodes 44 amino acid residues. A single silent mutation G66A in the NS2A gene of the JEV vaccine strain SA14-14-2, which abrogates the pseudoknot structure of the frameshift-stimulating motif, prevented NS1′ production in vitro and reduced neurovirulence [9]. NS1′ was shown to increase JEV replication in avian cells, which may facilitate the amplification/maintenance role of birds in the virus-transmission cycle in nature [10].

Research into RNA viruses has been advanced by the development of the reverse genetics system. However, the construction of an infectious cDNA clone for JEV, like other flavivirus, has been hampered by the genetic instability of cloned cDNAs propagated in *Escherichia coli* [11]. Many efforts have been made to overcome this instability of JEV cDNAs in bacteria. The earliest full-length infectious JEV clone was constructed in 1992 by in vitro ligation of two half-genomic cDNA fragments prior to RNA transcription [12]. Yamshchikov et al. [13] inserted an intron containing a stop codon into the 3′ end of the JEV core gene, which markedly stabilized a JEV infectious cDNA clone in bacteria. Some *E. coli* promoter (ECP) sequences have recently been identified in the JEV genome, and full-length infectious JEV cDNA clones were successfully propagated in *E. coli* by introducing an A-to-C mutation into the ECP sequences to reduce the bacterial promoter activity of virus genomes [14]. Subsequently, Pu et al. [15] introduced seven repeats of the tetracycline-response element and a minimal cytomegalovirus promoter upstream of the JEV genome to reduce the ECP activity of the JEV genome in bacteria, and developed a stable infectious JEV cDNA clone. Some low- and medium-copy-number vectors, such as bacterial artificial chromosomes (BACs) and pBR322, have been used to develop infectious cDNA clones for some flaviviruses [11, 16]. However, a stable, full-length cDNA for JEV has only been developed using a BAC vector [16].

We previously found that propagation at room temperature could markedly improve the stability of JEV cDNA clones. In this report, we developed an infectious, full-length cDNA clone, pJEHEN, of GI JEV strain HEN0701 by propagating cDNA clones at room temperature and using medium-copy-number pBR322 as a vector. The infectious cDNA clone pJEHEN was very stable and easy to manipulate, which will support future virology and pathogenesis studies, and aid vaccine development of GI JEV.

Materials and methods

Cells and viruses

BHK-21 cells (ATCC, CCL-10) were grown at 37 °C in Eagle's minimal essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % fetal bovine serum (FBS). Vero cells (ATCC) were grown at 37 °C in Dulbecco's modified eagle medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % FBS. GI JEV strain HEN0701 (GenBank Accession No. FJ495189) was isolated from the cerebrospinal fluid of aborted swine fetuses in Henan province, China in 2007.

Construction of full-length cDNA clone for HEN0701

A pBR322-derived vector pBR322M was constructed by replacing the *HindIII–Sal*I fragment in the tetracycline-resistance gene of pBR322 with a pair of complementary oligonucleotides to create the sequence 5′-AAGCTTC-CACGCGTCTACCTCGAGCTTGAGGATCCAGTCTAG ACCATGCGGCCTCGAC-3′, as described by Shi et al. [17]. This was then used to assemble the full-length cDNA of JEV. Four recombinant plasmids, pHJE1 (nucleotides (nt) 1–2913 of HEN0701 genome), pHJE2 (nt 2371–5675), pHJE3 (nt 5455–9204), and pHJE4 (nt 8791–10965), carrying overlapping cDNA fragments of the HEN0701 genome, were constructed as described previously [18]. T7 promoter sequences were added to the 5′ end of genomic cDNA by polymerase chain reaction (PCR) with the primers PJET7 and PJER1 (Table 1). Hepatitis delta virus (HDV) ribozyme sequences (HDVr) were added to the 3′ end of the genomic cDNA by PCR with the forward (PJEF4) and backward primers (PJEHDR1, PJEHDR2, and PJEHDR3) to generate a natural 3′ terminus during run-off transcription of the plasmid linearized at the 3′ end of the viral genome (Table 1). The resulting cDNA segments of the expected size were cloned into a pCR-Blunt-TOPO vector (Invitrogen, Carlsbad, CA, USA), generating pHT7JE1 and pHJE4-Hr. A full-length cDNA clone, pJE-HEN, for HEN0701 was then assembled with the two modified cDNA segments and two other cDNA segments located in the middle of the JEV genome by a series of subcloning steps, as shown in Fig. 1. First, the fragment JEF4-HDVr was cleaved from pHJE4-Hr with *Mlu*I plus *Xba*I and re-ligated into *Mlu*I- and *Xba*I-cleaved

Table 1 Primers used for PCR

| Primer	Sequence (5′-3′)	Purpose
PJET7	GTAATGCGGCCGCTAATACGACTCACTATAGAGAAGTTTATCTGTGTGAACTTCTTG	PCR for adding T7 sequences to 5′ end of JEV genome
PJER1	ATCAGGGCATTCCTTTGTCTCG	
PJEF4	GTCAAGGAAGTGCTCAACGAGAC	PCR for adding HDV ribozyme sequences to 3′ end of JEV genome
PJEHDR1	GAGGTGGAGATGCCATGCCGACCCAGATCCTGTGTTCTTCCTCACCAC	
PHDVR2	CGTCCTCCTTCGGATGCCCAGGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCC	
PHDVR3	CGCTCGAGCGCCCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTCGGATGCCCAG	
N2TAF	CAACGGCGAAATGATTGACCCATTTCAGCTGGGCCTTCTGGTG	Mutant PCR of T-to-A at nt 3558 of HEN0701 genome
N2TAR	CACCAGAAGGCCCAGCTGAAATGGGTCAATCATTTCGCCGTTG	
PJEF3322	GGAACAAAAGTCACCATCACAGAG	RT-PCR for amplifying NS2a of vJETA
PJER3927	CAGCGTTCAGGATTCCGTG	
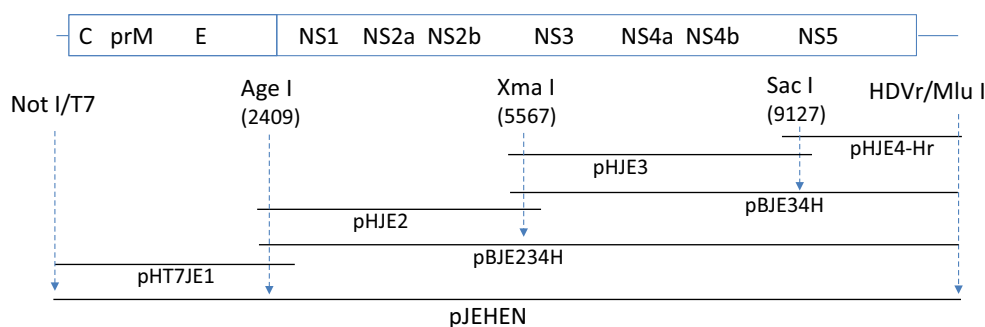
Fig. 1 Construction of full-length cDNA clone of JEV HEN0701. Genome organization, restriction enzymes, and nucleotide positions used for cloning are shown at the *top*. Four fragments were assembled to form the full-length cDNA clone of HEN0701 (pJEHEN) as described in "Materials and methods" section. The complete JEV cDNA was placed under the control of T7 promoter elements for in vitro transcription

pBR322M, yielding plasmid pBJE4H. Second, the fragment JEF3 was cleaved from pHJE3 with *Sac*I plus *Xba*I and re-ligated into pBJE4H digested with the same enzymes, yielding plasmid pBJE34H. Third, the fragment JEF2 was cleaved from pHJE2 with *Xma*I plus *Bam*HI and re-ligated into *Xma*I- and *Bam*HI-cleaved pBJE34H, yielding plasmid pBJE234H. Finally, the fragment T7-JEF1 was cleaved from pHT7JE1 with *Not*I plus *Age*I and re-ligated into pBJE234H digested with the same enzymes, yielding plasmid pJEHEN. Standard recombinant DNA procedures were followed, except that the recombinant clones were propagated at 23 °C.

Construction of mutant cDNA clone

The plasmid pHJE2 was used as the template for mutagenesis PCR. One pair of primers N2TAF/N2TAR (Table 1) containing the desired mutations was designed and synthesized based on HEN0701 genomic sequences. Quickchange PCR mutagenesis (Stratagene, Santa Clara, CA, USA) was carried out as instructed by the supplier. The PCR mixture was digested with DpnI to eliminate the pHJE2 template, and then transformed into TOP10-competent cells. The mutant plasmid pH3558TA was confirmed by nucleotide sequencing and the target region was swapped into the pJEHEN backbone treated with *Age*I plus *Xma*I, resulting in the full-length mutant clone pHENTA.

RNA transcription and transfection

For in vitro transcription, 2 μg of pJEHEN or pHENTA was linearized by cleavage with the restriction enzyme *Mlu*I, followed by recovery with a QIAquick PCR Purification kit (Qiagen, Germany). RNA transcripts were synthesized using an mMessage mMachine T7 Kit (Ambion Inc., Austin, TX, USA) using the purified linearized plasmid as a template in a 20 μl reaction mixture, according to the manufacturer's protocol. The reaction mixture was incubated at 37 °C for 2 h, followed by the addition of 1 μl DNase I to remove the DNA template. Two microliters of the RNA transcripts were analyzed on a 1 % agarose gel, and the RNA transcripts were stored in aliquots at −80 °C.

For RNA transfection, BHK-21 cells were grown to 75–85 % confluence in 35 mm culture dishes and transfected with the 1, 2, and 4 µg of RNA transcripts, respectively, using the DMRIE-C reagent according to the manufacturer's instructions (Invitrogen). After 6 h of exposure to DMRIE-C and RNA, the transfected cells were washed and fresh medium was added. The transfected cells were incubated at 37 °C under 5 % CO_2 and the JEV-specific cytopathic effect (CPE) was monitored daily. When the percentage of cytopathic cells exceeded 75 %, the supernatant was harvested and used to infect BHK-21 cells, generating a virus stock for use in further growth curves and virulence. This stock was stored in aliquots at −70 °C.

Immunofluorescence assay (IFA)

BHK-21 monolayers grown in 35 mm culture dishes were infected with rescued virus at a dilution of 1:500, or HEN0701 at a multiplicity of infection (MOI) of 0.01. After 1 h incubation at 37 °C, the monolayers were washed once with phosphate-buffered saline (PBS) before adding fresh medium. Cells were incubated at 37 °C for a further 2 days, washed once with PBS, followed by fixation in cold methanol for 10 min at −20 °C. Cells were then permeabilized with 0.1 % Tween-20 in PBS at room temperature for 5 min. After blocking with 2 % bovine serum albumin in PBS for 30 min at 37 °C, the cells were incubated for 1 h at 37 °C with anti-JEV NS3 protein monoclonal antibody (mAb) (kindly provided by Dr. Shengbo Cao, Huazhong Agricultural University, Wuhan, China) at 1:500 dilution in PBS plus 1 % bovine serum albumin. After washing, bound antibody was detected using goat anti-mouse IgG conjugated to Alexa Fluor 586 (Invitrogen).

Stability assay

pJEHEN (20 ng) was transformed into competent cells. The bacteria were then streaked onto LB agar and incubated at 23 °C. Three colonies were picked to grow in liquid LB medium. Three clones were isolated from the colonies using a QIAprep Spin Miniprep Kit (Qiagen) and transformed into competent cells again. Three clones after five rounds of plasmid–transformant–plasmid cycle were used for in vitro virus rescue.

Virus growth curve

BHK-21 cell monolayers grown in 35 mm culture dishes were infected with JEV at an MOI of 5 (one-step growth curve) or 0.01 (multiple-step growth curve). At 1 h post-infection (hpi), the monolayers were washed twice with PBS and 2 ml fresh medium (MEM plus 2 % FBS) was added. Infected cell supernatants (200 µl) were collected and the same volume of fresh medium was added at different time points (4–36 hpi for one-step and 12–96 hpi for multiple-step growth curve). Virus growth curves were determined from virus titers at different time points.

JEV titers were determined as the tissue culture infectious dose $(TCID)_{50}$/µl in BHK-21 cells. Serial tenfold dilutions of virus were prepared in MEM plus 2 % FBS and inoculated to BHK-21 monolayers in 96-well plates (100 µl/well). The plates were incubated for 4 days at 37 °C under 5 % CO_2. Titers in $TCID_{50}$/µl were calculated from the CPE according to the Reed–Muench formula.

Plaque assay

The viruses HEN0701, vJEHEN, and vJETA, were serially tenfold diluted in MEM, and 0.5 ml of the respective viruses were inoculated onto BHK-21 monolayers in 35 mm culture dishes. After 1 h at 37 °C, the monolayers were washed twice with PBS and overlaid with 4 ml MEM containing 1 % (w/v) low-melting-point agarose and 2 % FBS. After incubation for a further 4 days at 37 °C, plaques were stained using 5 % (w/v) crystal violet.

Reverse transcription-PCR

Viral RNA was extracted from 140 µl of the viral supernatant using a QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions. The purified viral RNA was used as template for cDNA synthesis with the primer PJER3927 (Table 1) and SuperScript reverse transcriptase (Invitrogen). PCR was carried out using PfuUltra Hotstart high-fidelity DNA polymerase (Stratagene) with a pair of primers (PJEF3322 and PJER3927) (Table 1). cDNA segments of the expected size were cloned into pCR-Blunt-TOPO and then transformed into the competent E. coli strain TOP10, following the manufacturer's instructions. Recombinant plasmids were confirmed by EcoRI digestion, and cDNA sequences of JEV were determined by Invitrogen Corporation (Shanghai, China) using a 3730 XL DNA Analyzer.

Western blotting

Vero cells were infected with HEN0701, vJEHEN, or vJETA at an MOI of 0.5 and then harvested with Cell-lysis Buffer (Beyotime Biotechnology, Nantong, China) containing 1 mM phenylmethanesulfonyl fluoride (Beyotime Biotechnology) at 24 hpi. Protein samples were heated to 100 °C for 5 min, run on 12 % sodium dodecyl sulfate–polyacrylamide gels, and transferred to nitrocellulose membranes. Membranes were blocked with 5 % milk–Tris-buffered saline supplemented with 0.1 % Tween

(TBS-T) overnight at 4 °C and then incubated with anti-JEV NS1 protein mAb (kindly provided by Dr. Ronghong Hua, Harbin Veterinary Research Institute, Harbin, China) for 1 h at 37 °C. After several washes in TBS-T, membranes were incubated with 1:10,000 anti-mouse IgG conjugated to horseradish peroxidase (Sigma-Aldrich) for 30 min at 37 °C. Signals were detected on radiographic film using a chemiluminescent kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Mouse neurovirulence testing

Three-week-old White Kunming mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., housed in an environmentally controlled room, and maintained on standard laboratory feed and water ad libitum throughout the study. HEN0701, vJEHEN, and vJETA were serially tenfold diluted in MEM, respectively. Five mice per group were inoculated intracerebrally with 50 μl diluent virus and then monitored for 3 weeks. Dead mice were recorded daily. The lethal dose $(LD)_{50}$/ml for each virus was calculated according to the Reed–Muench formula. The animal experiments in this study were approved by the Animal Care and Ethics Committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, and were carried out in accordance with the conventional animal-welfare regulations and standards.

Results

Generation of an infectious, full-length cDNA clone pJEHEN

A full-length cDNA clone, pJEHEN, for HEN0701 was assembled by a series of subcloning steps, as shown in Fig. 1. The pJEHEN carried the full-length cDNA of JEV HEN0701 under the control of the T7 promotor. Sequence analysis confirmed that the JEV cDNA sequences in pJEHEN were the same as the sequences in the HEN0701 genome (FJ495189).

After pJEHEN was linearized, capped RNA transcripts were synthesized from the linearized pJEHEN. The RNA transcripts showed a single band on a 1 % agarose gel (Fig. 2).

BHK-21 cells were transfected with RNA transcripts. CPE was detected in RNA-transfected BHK-21 cells at 72-h post-transfection, while no CPE was detected in the RNase-free, water-transfected control (Fig. 3). The cell-culture supernatant was harvested and an aliquot was inoculated onto fresh BHK-21 cells. A pronounced CPE was detected in BHK-21 cells inoculated with supernatant

Fig. 2 RNA transcripts of linearized pJEHEN and pHENTA. RNA transcripts (2 μl) derived from linearized pJEHEN (*lane 1*) and pHENTA (*lane 2*) were analyzed on a 1.0 % agarose gel
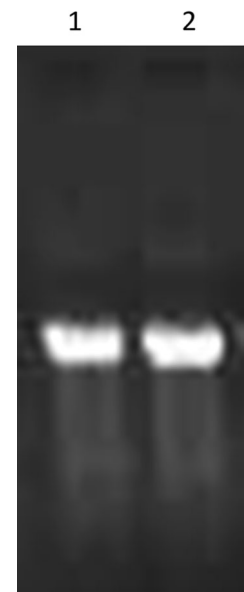
derived from 4 μg-RNA-transfected cells at 24 hpi, and supernatant derived from 1 μg-RNA-transfected cells at 36 hpi. No CPE was detected in negative control cells transfected with RNase-free water at 5 days post-inoculation. To test the specificity of the rescued viruses, IFA was carried out. BHK-21 cells infected with either vJEHEN or HEN0701 showed positive staining with mAb directed against JEV NS3 protein (Fig. 4). These results suggest that infectious JEV, vJEHEN, was generated from pJEHEN.

Stability of pJEHEN

We investigated the stability of pJEHEN in three clones selected at random and subjected to five rounds of plasmid–transformant–plasmid cycle. The infection of the passage plasmids was tested by transfection of 2 μg RNA transcribed in vitro from three passage clones into BHK-21 cells, respectively. Infectious JEV was generated from all three clones, indicating that pJEHEN was stable.

Generation of mutant virus vJETA

To rescue mutant virus, RNA transcribed in vitro from pHENTA was transfected into BHK-21 cells. CPE was detected in the RNA-transfected BHK-21 cells at 96 h post-transfection. BHK-21 cells infected with the supernatant of cells with CPE also displayed positive staining with mAb directed against JEV NS3 protein (Fig. 4), confirming that the supernatant contained infectious JEVs. To confirm that the mutant virus, vJETA, containing the expected mutation, reverse transcription-PCR was conducted as described above using RNAs from the mutant virus. Purification of the amplified products and nucleotide
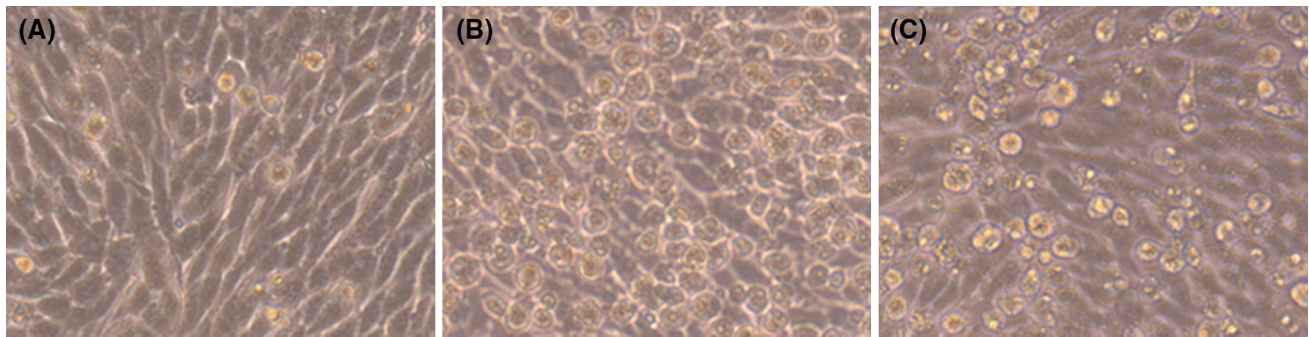
Fig. 3 JEV-specific cytopathic effect (CPE) of rescued viruses. BHK-21 cells were transfected with RNA transcripts from pJEHEN. The CPE were observed at 72 h after transfection. Cells were observed used a fluorescence microscope under natural light. **a** Negative control. **b** HEN0701 **c** vJEHEN
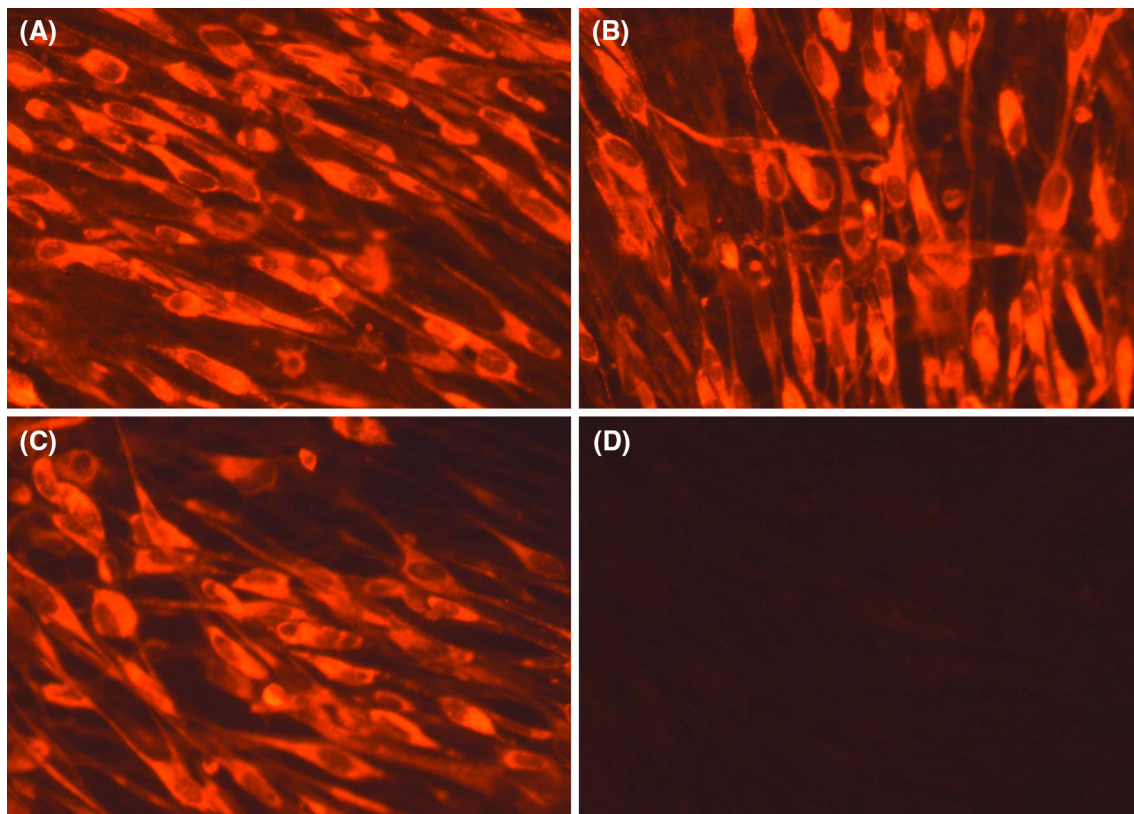

Fig. 4 Immunofluorescence analyses of rescued viruses. BHK-21 cells were infected with HEN0701, rescued vJEHEN, or vJETA. At 48 hpi, the infected cells were subjected to immunofluorescence assay using anti-JEV NS3 monoclonal antibody. Cells were observed at under a fluorescence microscope. **a** HEN0701. **b** vJEHEN. **c** vJETA. **d** Negative control

sequencing showed that only one nucleotide at nt 3558 of the JEV genome differed between vJETA and HEN0701 (Fig. 5). T was replaced by A at nt 3558 in the vJETA genome. vJETA thus contained the designed mutation. To examine the effect of the T-to-A mutation on the expression of NS1′ protein, we infected Vero cells with vJETA, vJEHEN, or HEN0701, and subjected the cell lysates to western blotting using the JEV NS1-specific mAb. Both NS1 and NS1′ were detected in cells infected with HEN0701 or vJEHEN, whereas only NS1 was detected in vJETA-infected cells (Fig. 6). This showed that the T-to-A mutation at nt 3558 of the HEN0701 genome abolished the expression of NS1′.

Growth characteristics of cloned virus vJEHEN and mutant virus vJETA

The cloned virus vJEHEN, mutant virus vJETA, and parental virus HEN0701 were inoculated onto BHK-21 cells at an MOI of 5 or 0.01. One-step and multiple-step growth
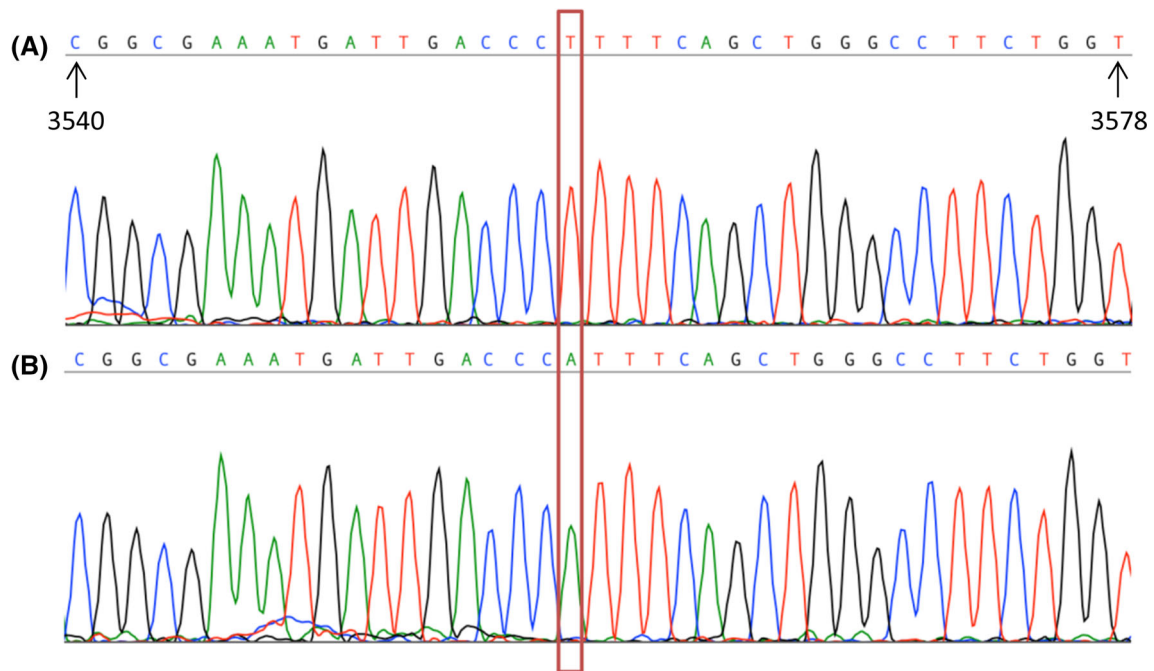
Fig. 5 T-to-A mutation in the conserved slippery heptanucleotide. Sequences of **a** HEN0701, and **b** vJETA. The T-to-A mutation in the NS2a gene of vJETA is indicated by the frame. Nucleotide positions were numbered according to the sequence of the HEN0701 genome
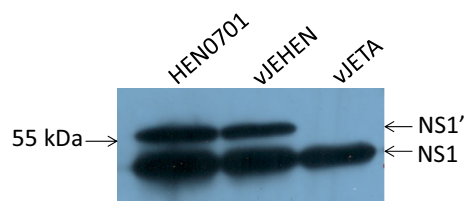

Fig. 6 Expression of NS1 and NS1′ in JEV-infected cells. Vero cells were infected with HEN0701, vJEHEN, or vJETA, and western blotting assays were performed using JEV NS1-specific mAb

curves for vJEHEN, vJETA, and HEN0701 were determined (Fig. 7a). The growth kinetics of vJEHEN were identical to those of HEN0701 in both one-step and multiple-growth curves. Compared with vJEHEN and HEN0701, vJETA grew more slowly on BHK-21 cells and the vJETA titer was consistently lower than those of HEN0701 and vJEHEN. There was no difference in plaque morphology on BHK-21 cells between vJEHEN and HEN0701, though vJETA exhibited smaller plaques than the other viruses (Fig. 7b). These results revealed that the cloned vJEHEN and its parental virus were indistinguishable in terms of replication and spread in BHK-21 cells. A T-to-A mutation retarded the replication and spread of vJETA in BHK-21 cells.

Virulence in mice

To determine the neurovirulence of vJETA, vJEHEN, and HEN0701, 3-week-old mice were inoculated

intracerebrally with 50 µl virus diluent. Mice died at 6–9 days post-inoculation for vJEHEN and HEN0701, compared with 7–10 days for vJETA (Fig. 8). The $\log_{10}$ plaque-forming unit/LD_{50} values for vJEHEN and HEN0701 were 0.33 and 0.29, respectively, compared with 228.54 for vJETA. These results suggest that the cloned virus, vJEHEN, had similar virulence to HEN0701 in mice, while the T-to-A mutation markedly decreased the neurovirulence of JEV.

Discussion

We previously cloned the genome of JEV strain HEN0701 using four overlapping cDNA fragments [18]. In the current study, we developed an infectious cDNA clone, pJEHEN, of JEV HEN0701 strain based on these four cDNA fragments. We added T7 promoter sequences to the 5′ end of HEN0701 genomic cDNA to launch the RNA transcripts in vitro, and then added HDVr sequences to the 3′ end to generate a natural 3′ terminus of JEV. During the development of infectious flavivirus cDNA clones, we used specific restriction endonucleases to linearize full-length infectious cDNA clones so that the 3′-terminus sequences of the linearized full-length cDNA were as close as possible to the authentic 3′ end [13, 14, 16, 17]. Mung bean nuclease has been used to delete the non-viral nucleotides at the 3′ terminus of linearized full-length JEV cDNA, and the RNA transcripts derived from linearized full-length
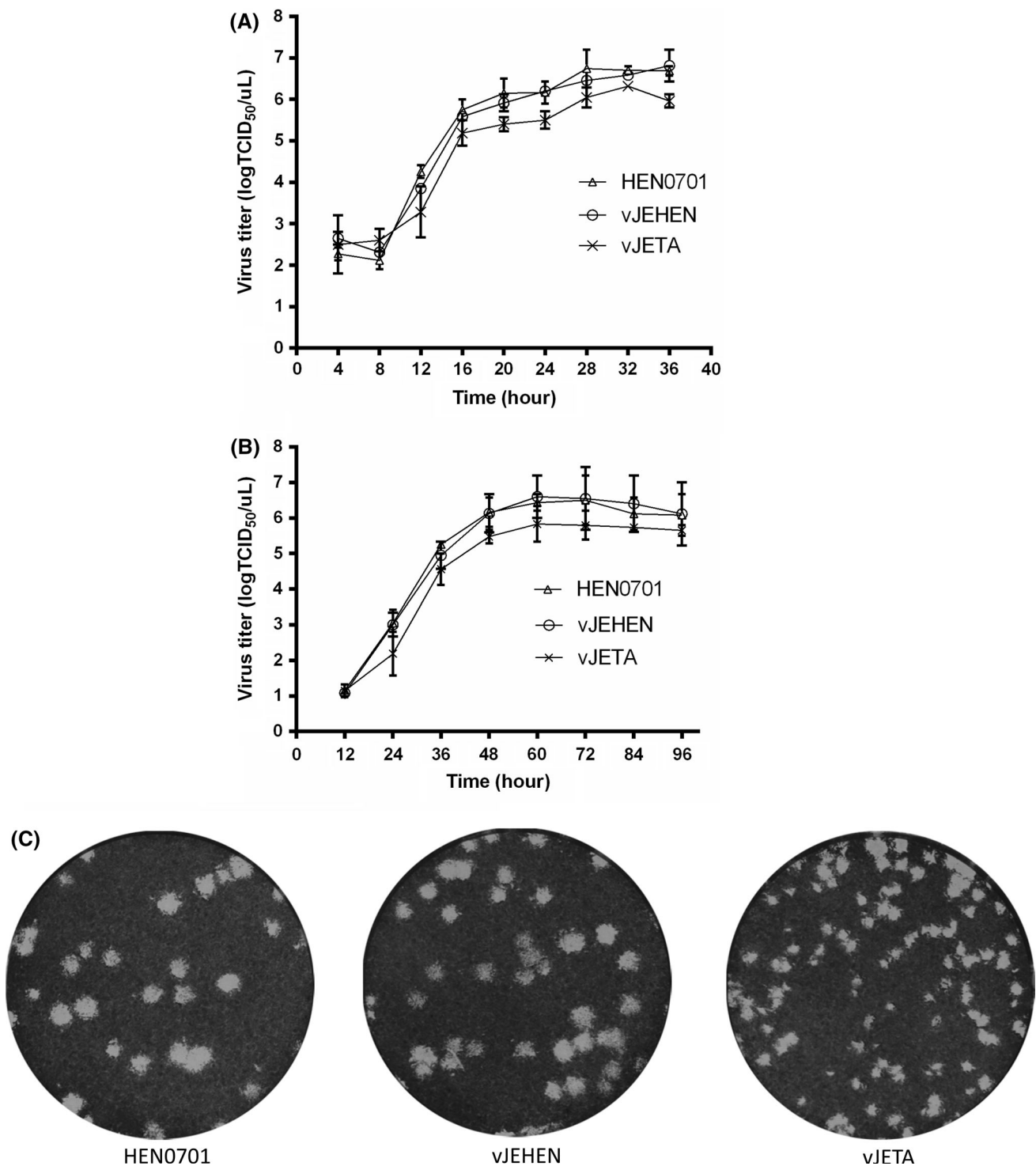
Fig. 7 Growth properties of vJEHEN and vJETA. **a** One-step growth curves of vJEHEN and vJETA in BHK-21 cells. **b** Multiple-step growth curves of vJEHEN and vJETA in BHK-21 cells. **c** Plaque morphology of vJEHEN and vJETA in BHK-21 cells

JEV cDNA showed increased infectivity after this modification [16]. The genomic ribozyme of human HDV was added to the 3′ end of the infectious full-length cDNAs to generate a natural viral 3′ end [15, 19]. Numerous restriction endonucleases may thus be used to linearize an infectious cDNA clone. Finally, the two modified cDNA fragments and two middle fragments were assembled into a full-length genomic cDNA in the vector pBR322 using conventional recombinant DNA procedures, except that the recombinant clones were propagated at room temperature.
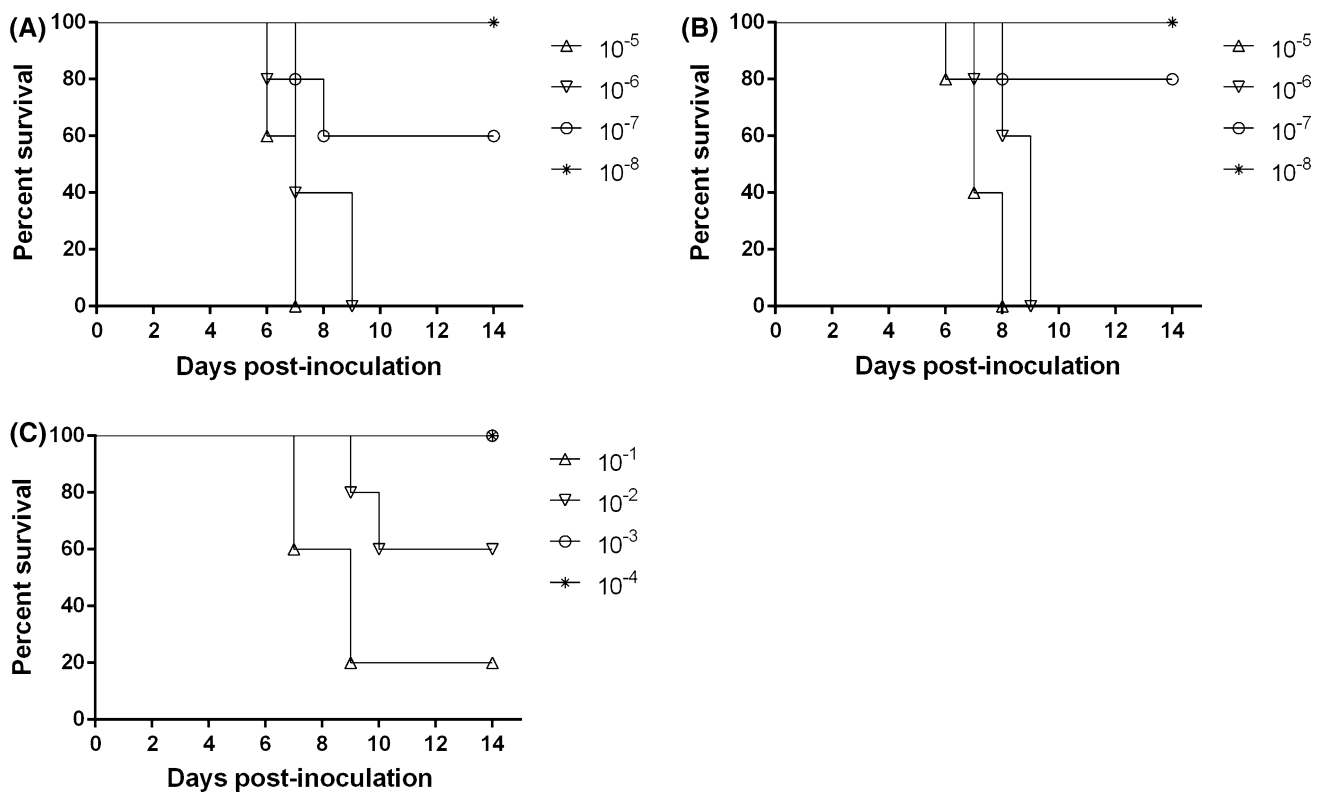
Fig. 8 Neurovirulence was tested by intracerebral inoculation of 3-week-old KM mice. Mice were infected with HEN0701, vJEHEN, and vJETA at four different dilutions, respectively. **a** HEN0701. **b** vJEHEN. **c** vJETA

Infectious JEV was generated after transfection with RNA transcripts derived from linearized pJEHEN. The rescued JEV, vJEHEN, showed the same growth properties in vitro and similar neurovirulence to its parent strain HEN0701. In addition, RNA transcripts derived from the passage pJE-HEN after five rounds of plasmid–transformant–plasmid cycle efficiently generated infectious JEV. These data confirmed the genetic stability of pJEHEN.

A full-length infectious JEV cDNA clone was previously developed in a BAC vector [16]. However, output efficiency of BACs is very low and BAC vectors contain numerous restriction enzyme recognition sites, which is a disadvantage in terms of manipulating the viral cDNA in the BAC vector. Pu et al. [14] recently reported the presence of ECP sequences in JEV genomic cDNA, and mutation of these sequences reduced ECP activity and enhanced the stability of the cDNA clone in *E. coli*. They developed an infectious JEV cDNA clone that was stably propagated in *E. coli* by an A-to-C mutation at nt 90 of the JEV genome. In a previous study, we also found that the 5′-end sequences of the JEV genome contained an ECP. At 37 °C, this sequence promoted expression of the JEV structural polyprotein, which was harmful to host *E. coli*, resulting in instability of JEV cDNA clones. However, ECP activity was markedly decreased at room temperature. Sriburi et al. [20] developed an infectious, full-length clone

of DENV strain 16681 in a high-copy-number pBluescript II KS vector propagated at room temperature (20–25 °C). We attempted to assemble four overlapping cDNA fragments of the HEN0701 genome into a full-length clone of JEV in the pBluescript II SK vector. Although the full-length clone was propagated at room temperature, the subsequent RNA transcripts did not generate infectious JEV. Sequencing the clone revealed nucleotide deletions in the coding region for E and NS1 (data not shown). It is possible that the instability of full-length cDNA clones differs among flaviviruses. Pu et al. [15] showed that five repeats of the GAL4 upstream activating sequence or five repeats of BamHI linkers upstream of the viral genome could stabilize infectious, full-length cDNA clones of DENV2, but not JEV, implying that full-length JEV cDNA was less stable in *E. coli* than DENV cDNA. The medium-copy-number vector pBR322 is usually used to carry the full-length infectious cDNA clones of flaviviruses such as WNV, tick-borne encephalitis virus, yellow fever virus, and DENV [11, 17]. Yun et al. [16] unsuccessfully tried to use a range of vectors, including high-copy-number pUC-derived, medium-copy-number pBR322-derived, and low-copy-number pACYC184-derived vectors, to assemble a full-length JEV cDNA. However, in the current study, we propagated stable, infectious, full-length cDNA of HEN0701 in pBR322 at room temperature for the effective

generation of infectious JEV, indicating that growth at room temperature combined with the use of pBR322 provides a simple and effective method for developing infectious JEV cDNA clones. A nucleotide mutation was introduced in subclone pHJE2 by site-directed PCR mutagenesis, and substituted into the infectious full-length cDNA to generate mutant virus. This demonstrated the ability to manipulate the infectious clone pJEHEN.

NS1′ protein of JEV serogroup flaviviruses is produced as a result of a −1 ribosomal frameshift at the conserved slippery heptanucleotide (YCCUUUU) and 3′ adjacent potential pseudoknot located at the 5′ end of the NS2A gene [7, 8]. In the current study, the third nucleotide U of the slippery heptanucleotide (CCU UUU) was mutated into A (CCA UUU), thus destroying the conserved slippery sequence. The rescued mutant virus vJETA did not express NS1′ in mammalian cells and the neurovirulence and growth rate of vJETA were markedly decreased compared with its parent strain HEN0701 and vJEHEN. However, abolishing NS1′ had no effect on viral growth in mammalian cells [9, 10]. The U-to-A mutation in the slippery sequences changed an NS2a codon from CCU to CCA, which is a synonymous mutation. However, the role of this synonymous mutation in the slow growth of vJETA in mammalian cells is unclear.

In conclusion, we developed a simple method of generating an infectious JEV cDNA clone using medium-copy-number pBR322 as a vector and propagating the clones at room temperature. The resulting full-length infectious cDNA clone pJEHEN was very stable and convenient to manipulate, and will thus be of use in future viral replication and pathogenesis studies, as well as in vaccine development.

Compliance with ethical standards

Conflict of interest All individual participants approve to submit and declare no conflict of interests.

Ethical approval The animal experiments in this study were approved by the Animal Care and Ethics Committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, and were carried out in accordance with conventional animal-welfare regulations and standards.

References

1. G.L. Campbell, S.L. Hills, M. Fischer, J.A. Jacobson, C.H. Hoke, J.M. Hombach, A.A. Marfin, T. Solomon, T.F. Tsai, V.D. Tsu, A.S. Ginsburg, Estimated global incidence of Japanese encephalitis: a systematic review. Bull. World Health Organ. **89**(10), 766–774 (2011)

2. D. Ghosh, A. Basu, Japanese encephalitis-a pathological and clinical perspective. PLoS Negl. Trop. Dis. **3**, e437 (2009)

3. T.P. Endy, A. Nisalak, Japanese encephalitis virus: ecology and epidemiology. Curr. Top. Microbiol. Immunol. **267**, 11–48 (2002)

4. J.S. Mackenzie, A.D. Barrett, V. Deubel, The Japanese encephalitis serological group of flaviviruses: a brief introduction to the group. Curr. Top. Microbiol. Immunol. **267**, 1–10 (2002)

5. T.J. Chambers, C.S. Hahn, R. Galler, C.M. Rice, Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. **44**, 649–688 (1990)

6. P.W. Mason, Maturation of Japanese encephalitis virus glycoproteins produced by infected mammalian and mosquito cells. Virology **169**, 354–364 (1989)

7. A.E. Firth, J.F. Atkins, A conserved predicted pseudoknot in the NS2A-encoding sequence of West Nile and Japanese encephalitis flaviviruses suggests NS1′ may derive from ribosomal frameshifting. Virol. J. **6**, 14 (2009)

8. E.B. Melian, E. Hinzman, T. Nagasaki, A.E. Firth, N.M. Wills, A.S. Nouwens, B.J. Blitvich, J. Leung, A. Funk, J.F. Atkins, R. Hall, A.A. Khromykh, NS1′ of flaviviruses in the Japanese encephalitis virus serogroup is a product of ribosomal frameshifting and plays a role in viral neuroinvasiveness. J. Virol. **84**, 1641–1647 (2010)

9. Q. Ye, X.F. Li, H. Zhao, S.H. Li, Y.Q. Deng, R.Y. Cao, K.Y. Song, H.J. Wang, R.H. Hua, Y.X. Yu, X. Zhou, E.D. Qin, C.F. Qin, A single nucleotide mutation in NS2A of Japanese encephalitis-live vaccine virus (SA14-14-2) ablates NS1′ formation and contributes to attenuation. J. Gen. Virol. **93**, 1959–1964 (2012)

10. Y. Takamatsu, K. Okamoto, D.T. Dinh, F. Yu, D. Hayasaka, L. Uchida, T. Nabeshima, C.C. Buerano, K. Morita, NS1′ protein expression facilitates production of Japanese encephalitis virus in avian cells and embryonated chicken eggs. J. Gen. Virol. **95**, 373–383 (2014)

11. N. Ruggli, C.M. Rice, Functional cDNA clones of the Flaviviridae: strategies and applications. Adv. Virus Res. **53**, 183–207 (1999)

12. H. Sumiyoshi, C.H. Hoke, D.W. Trent, Infectious Japanese encephalitis virus RNA can be synthesized from in vitro-ligated cDNA templates. J. Virol. **66**, 5425–5431 (1992)

13. V. Yamshchikov, V. Mishin, F. Cominelli, A new strategy in design of +RNA virus infectious clones enabling their stable propagation in E. coli. Virology **281**, 272–280 (2001)

14. S.Y. Pu, R.H. Wu, C.C. Yang, T.M. Jao, M.H. Tsai, J.C. Wang, H.M. Lin, Y.S. Chao, A. Yueh, Successful propagation of flavivirus infectious cDNAs by a novel method to reduce the cryptic bacterial promoter activity of virus genomes. J. Virol. **85**, 2927–2941 (2011)

15. S.Y. Pu, R.H. Wu, M.H. Tsai, C.C. Yang, C.M. Chang, A. Yueh, A novel approach to propagate flavivirus infectious cDNA clones in bacteria by introducing tandem repeat sequences upstream of virus genome. J. Gen. Virol. **95**, 1493–1503 (2014)

16. S.I. Yun, S.Y. Kim, C.M. Rice, Y.M. Lee, Development and application of a reverse genetics system for Japanese encephalitis virus. J. Virol. **77**, 6450–6465 (2003)

17. P.Y. Shi, M. Tilgner, M.K. Lo, K.A. Kent, K.A. Bernard, Infectious cDNA clone of the epidemic west Nile virus from New York city. J. Virol. **76**, 5847–5856 (2002)

18. H. Zheng, T. Shan, Y. Deng, C. Sun, S. Yuan, S. Yin, G. Tong, Molecular characterization of Japanese encephalitis virus strains prevalent in Chinese swine herds. J. Vet. Sci. **14**, 27–36 (2013)

19. X. Qi, Y. Gao, H. Gao, X. Deng, Z. Bu, X. Wang, C. Fu, An improved method for infectious bursal disease virus rescue using RNA polymerase II system. J. Virol. Methods **142**, 81–88 (2007)

20. R. Sriburi, P. Keelapang, T. Duangchinda, S. Pruksakorn, N. Maneekarn, P. Malasit, N. Sittisombut, Construction of infectious dengue 2 virus cDNA clones using high copy number plasmid. J. Virol. Methods **92**, 71–82 (2001)