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SHORT REPORT

Development of a reverse genetics system for Japanese encephalitis virus strain SA14-14-2

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Abstract

Japanese encephalitis virus SA14-14-2 (JEV SA14-14-2) is a widely used vaccine in China and other southeastern countries to prevent Japanese encephalitis in children. In this study, a stable infectious cDNA clone of JEV SA14-14-2 with a low copy number pACYC177 vector dependent on the T7 promoter and T7 terminator was developed. Two introns were inserted into the capsid gene and envelope gene of JEV cDNA for gene stability. Hepatitis delta virus ribozyme (HDVr) was engineered into the 3′ UTR cDNA of JEV for authentic 3′ UTR transcription. The rescued virus showed biological properties indistinguishable from those of the parent strain (JEV SA14-14-2). The establishment of a JEV SA14-14-2 reverse genetics system lays the foundation for the further development of other flavivirus vaccines and viral pathogenesis studies.

Keywords Japanese encephalitis virus · Infectious clone · Intron · T7 terminator

Introduction

Japanese encephalitis (JE) is a vector-borne zoonosis that poses a threat to 4 billion people in Asia and people infected present clinical symptoms, including severe fever, seizure, and cognitive disorders [1]. Yearly, approximately 67,900 JE cases occur in JE-endemic countries [2]. JE is not only prevalent in the human population but also epidemic in pigs and water fowl. The pathogen is the Japanese encephalitis

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virus (JEV), a member of the *Flaviviridae* family [3]. JEV is a positive, single-stranded RNA virus with a genome of approximately 11,000 nt, including a single open reading frame encoding three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [4]. The following are five genotypes of JEV: GI, GII, GIII, GIV, and GV. JEV SA14-14-2 is a robust live-attenuated vaccine that belongs to genotype GIII and has been widely used for JE prevention in mainland China since 1989 [5].

The establishment of a reverse genetic system for the JEV SA14-14-2 is critical in the study of viral pathogenesis and vaccine development. Two main strategies are used to recover the flavivirus. In the first strategy, permissive cells are transfected with genomic RNA produced by in vitro transcription using the bacteriophage (T7 or SP6) promoter [6–13]. In the second strategy, permissive cells are directly transfected with viral cDNA using the eukaryotic promoter. The full-length cDNA of JEV is flanked by the eukaryotic cytomegalovirus (CMV) promoter, HDVr, and simian virus 40 polyadenylation signals (SV40 pA) or bovine growth hormone transcriptional terminator (BGH terminator) for virus recovery [14–16]. Other flaviviruses, such as Yellow fever virus [17] and Zika virus [18] also rescued virus in this way. Directly transfecting CMV promoter-launched infectious clones could simplify the procedure. In this study, we developed a T7 promoter-launched infectious clone of JEV, i.e., pFLJEV, on a low copy number vector pACYC177. A T7 terminator sequence (Table 1) and BSR T7/5 cells (BHK derivative that could express T7 RNA polymerase [19]) were used in order to overcome the need for vector linearization and in vitro transcription.

The full-length cDNA of JEV was divided into five fragments for assembly (Fig. 1). The T7 promoter (Table 1) derived from the pET-32a (+) vector was introduced prior to the 5′ UTR of the JEV cDNA. HDVr (Table 1) was added after the 3′ UTR of the JEV cDNA to generate an authentic 3′ end [20]. The linker covering the unique restriction sites and T7 terminator was synthesized by Sangon Biotech Co., Ltd. (Shanghai) and cloned into a *Bam*H I-linearized pACYC177 using the In-Fusion HD Cloning System (TaKaRa, Dalian, Liaoning, China). Due to the instability of JEV cDNA in *Escherichia coli (E. coli.)*, two introns were introduced. Fragment F2 with an intron (F2-intron) after 2217 bp was artificially synthesized (Sangon, Shanghai, China). Fragment F1 with an intron (F1-intron) after 356 bp was synthesized through overlap PCR using primers listed in Table 2; simultaneously, GAACTT was silently mutated into GAgCTc (Fig. 1) by the primer X2-F and the primer X3-F (Table 2) for precise excision. To maintain the authentic 3′ UTR, an HDVr sequence was added to the 3′ end of the JEV cDNA. Fragment F5-HDVr was synthesized through PCR as follows. The first 38 bp of this fragment was synthesized using primers F5-F and F5-R1. Then the product of this first PCR was used as the template of a second PCR using primers F5-F and F5-R2 (Table 2) in order to rescue the complete F5-HDVr fragment. Simultaneously, a silent mutation was introduced into fragment 5 by primer F5-F for differentiation from the parent virus (JEV SA14-14-2, cultured in the BHK-21 cells). The five fragments, i.e., F5-HDVr, F4, F3, F2-intron, and T7pro-F1-intron, were assembled in order into pACYC177-linker by a series of subcloning steps (Fig. 1). Each subclone and full-length JEV cDNA (pFLJEV) were sequenced. The clones were transformed into *E. coli* HB101 competent cells (TaKaRa, Dalian, Liaoning, China) for plasmid propagation, and the bacteria were cultured at 30 °C to decrease the gene mutation and deletion.

To generate the rescued virus, 5 μg pFLJEV was transfected into BSR T7/5 cells in 6-well plates with Lipofectamine 3000 (Thermo Scientific, Waltham, MA, USA) for the progeny virus recovery. The cells were incubated for 4–6 days at 37 °C and 5% CO_2, and the culture supernatants were collected at 24-h intervals and were inoculated in BSR T7/5 cells for indirect immunofluorescence assay (IFA) detection. A 1:20 diluted Anti-Japanese encephalitis virus E glycoprotein monoclonal antibody JE1 (IgG2a)

Table 1 Sequence elements in the JEV cDNA clones

Sequence elements	Sequences (5′–3′)	Source
T7 promoter	TAATACGACTCACTATAG	pET32a(+)
T7 terminator	TAGCATAACCCCTTGGGGCCTCTA AACGGGTCTTGAGGGGTTTTTTG	pET32a(+)
Intron	GTAAGTATCAAGGTTACAAGGCAG GTTTAAGGAGACCAATAGAAACTG GGCTTGTCGAGACAGAGAAGACTC TTGCGTTTCTGATAGGCACCTATTG GTCTTACTGACATCCACTTTGCCTT TCTCTCCACAG	GenBank accession: KX576684.1
HDVr	GGGTCGGCATGGCATCTCCACCTCC TCGCGGTCCGACCTGGGCATCCGAA GGAGGACGCACGTCCACTCGGATGG CTAAGGGAGGGCG	Inoue K. et al. J. Virol. Methods (2003) [17]

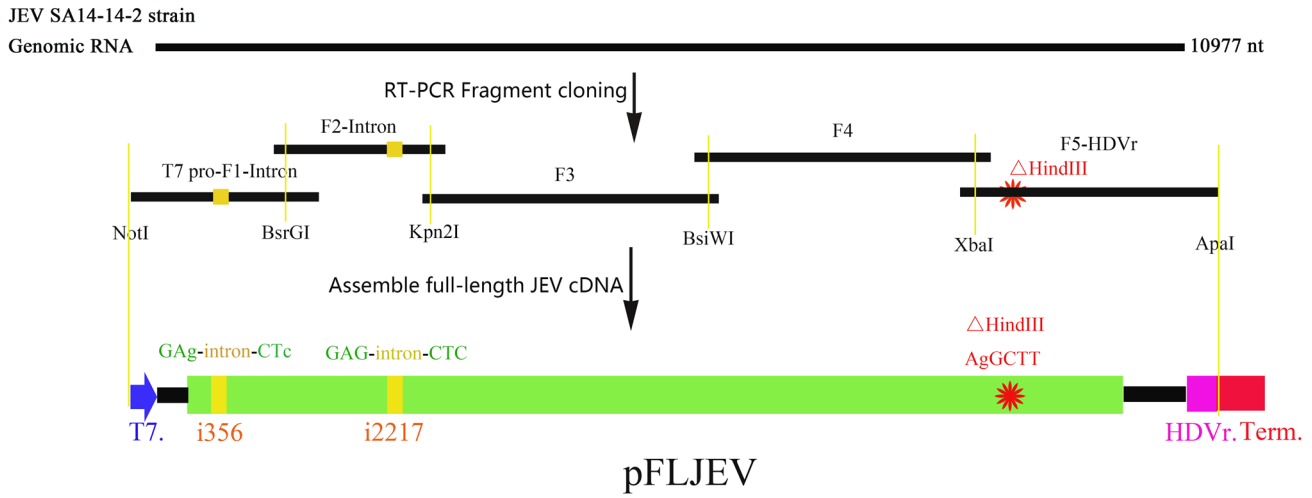

Fig. 1 Construction of the full-length cDNA of JEV SA14-14-2 (pFLJEV). A T7 promoter (blue arrow) was introduced prior to the 5′ UTR of the JEV cDNA. Two silent mutations (shown in lowercase) in fragment F1 were engineered to create a *Sac* I site for the insertion of introns. Introns (yellow bars) were inserted into F1 and F2 (see the text for details). A silent mutation (red star), which allows the distinction between the parent and rescued virus, was introduced in fragment F5-HDVr. In order to obtain an authentic 3′ end, the HDVr sequence (violet bar) was introduced just after the 3′ UTR

Table 2 Primers used for the amplification of JEV infectious cDNA clones

Primer names[a]	Primer sequences (5′–3′)[b]	Restriction endonucleases	Amplified fragments
F1-T7pro-F[c]	TCTGCGGCCGCtaatacgactcactat agAGAAGTTTATCTGTGTGAACTTCTT	*Not* I	T7pro-F1
F1-T7pro-R	CGAGAATTTTTCTGTACACATGCCA	*Bsr*G I	
F2-F	TGGCATGTGTACAGAAAAATTCTCG	*Bsr*G I	F2-Intron
F2-R[d]	TAACAGGTCTGATTTCCATTCCGTA	*Kpn*2 I	
F3-F	AAAACCAGGGACCTTGGGATG	*Kpn*2 I	F3
F3-R[d]	GCGACCGAGCACCTCTATGA	*Bsi*W I	
F4-F	ATGGGCCGCGTACGAATGCC	*Bsi*W I	F4
F4-R[d]	CAGCGATGTTCGTGAAAGTGTT	*Xba* I	
F5-F[e]	GTATCTAGAGTTTGA**GG**CTTGGGGTTCCTG	*Xba* I *Hin*d III	F5-HDVr
F5-R1	GGTCGGACCGCGAGGAGGTGGAGATGCCATGCCGACCCAGATCCTGTGTTCTTC CTCACCACC		
F5-R2	TTAGGGCCCCGCCCTCCCTTAGCCATCCGAGTGGACGTGCGTCCTCCTTCGGAT GCCCAGGTCGGACCGCGAGGAGGTGGAGAT	*Apa* I	
X1-F	TCTGCGGCCGCTAATACG		
X1-R[f]	CTCTCGTTTGAAACTAGTAAGATGT		
X2-F[f]	TCTTACTAGTTTCAAACGAGAGGTAAGTATCAAGGTTACAAGGC		
X2-R	CTGTGGAGAGAAAGGCAAAG		
X3-F[f]	CTTTCTCTCCACAGCTCGGAACACTCATTG		
X3-R	ATTCGTACGTGATAGTGTCCTCACACATGT		

[a]F and R indicate forward and reverse primers, respectively

[b]Restriction endonuclease sites are underlined

[c]Lowercase sequence represents the T7 promoter

[d]Restriction endonuclease sites without underlines are located in the sequence (not on primers)

[e]**AG**GCTT represents a knockout of *Hin*d III in the JEV cDNA by a silent A to **G** substitution

[f]Underlined letters represent the silent mutation of GAACTT to GAgCTc by primers

(Abcam, Cambridge, MA, USA) and 1:200 diluted FITC labeled Goat-anti-Mouse IgG (H+L) secondary antibody (Thermo Scientific, Waltham, MA, USA) were incubated to detect the specific fluorescence. The results indicated that the BSR T7/5 cells infected with the supernatants at 96 h and 120 h exhibited obvious fluorescence (Fig. 3a). Clear cytopathic effect (CPE) was demonstrated in the BSR T7/5 cells 120 h post transfection (data not shown). The titer was $10^{3.75}$ $TCID_{50}$/mL as measured using the Reed–Muench method. To increase the viral titer, the rescued virus was serially propagated in BSR T7/5 cells for three passages after which no mutation in the genome was detected through Sanger DNA sequencing. The rescued virus titer could reach $10^{7.5}$ $TCID_{50}$/mL.

To acquire the complete sequence of JEV SA14-14-2, 11 pairs of primers (Table 3) covering the whole genome were designed according to the JEV complete sequence (Accession number: AF315119) in the GenBank database. The viral RNA of JEV SA14-14-2 was extracted according to the viral RNA extraction kit protocol (Bioflux, Hangzhou, Zhejiang, China) and amplified through RT-PCR using the designed primers (Table 3) resulting in 11 overlapping fragments that were sequenced through Sanger DNA sequencing. The complete genomic sequence of the parent strain had been submitted to the National Center for Biotechnology Information (NCBI), and the GenBank accession number is MK585066. The differences between the parent and the rescued strains had been presented in the Supplementary material (Table S1).

The genetic marker and introns (removed from the rescued virus) were confirmed by an RT-PCR analysis of the relevant segments of fragment F1, F2, and F5 and sequenced by Sanger sequencing. The complete genomic sequence of the rescued virus had a sequence similar to that of the parent strain, except for a silent mutation at 9143 nt (Fig. 2a) and two silent mutations at 356 nt and 359 nt (Fig. 2b) (no amino acids changes). The introns at i356 bp and i2217 bp were removed (Fig. 2b) from the genome of the rescued virus by spliceosome mechanism [21]. Spliceosomes could recognize the 5′…GAG**GU…AG**CTC…3′ sequence (an abbreviated intron sequence is bold) to launch precise splicing. Researchers have acquired full-length cDNA clones and rescued progeny viruses (JEV and ZIKV) in this way [13, 14].

The BSR T7/5 cells were infected separately with the parent strain and the rescued virus at a multiplicity of infection (MOI) of 0.25. Both strains showed similar CPE after 48 h (Fig. 2c). The phenotypes of the parent and rescued virus were compared by a plaque assay. In order to do that,

Table 3 Primers used for the sequencing of full-length JEV cDNA

Primers[a]	Primer sequences (5′–3′)	Fragment location, nt
JEV-1F	AGAAGTTTATCTGTGTG	1–1216
JEV-1R	GCTTCTCCAGTCGTGGGG	
JEV-2F	GGCTTACAGTTTTAATTGTCTGGGA	968–2226
JEV-2R	GTCTTTGAGCTCCCTTCAAAGTTG	
JEV-3F	CAACTCAAAGGTGCTGGTC	2075–3228
JEV-3R	TTGGTCCGGCTATGGT	
JEV-4F	ATTGGCTAACTCCACATTTGTCGTA	2858–4066
JEV-4R	CCTATGACGAGGAGGATGATTCTGT	
JEV-5F	CACCTTGCTTTGATTGCTGT	3759–4948
JEV-5R	CCTGGTTTTGTCTGGATGTTTA	
JEV-6F	TAGAGGGATTCTTGGCACTT	4688–5947
JEV-6R	GGGTTTCCGAGGATGACT	
JEV-7F	GGGGAAAAAGGTCATCCAACT	5744–6987
JEV-7R	GTCCTGATGCCTGCGTCTTT	
JEV-8F	AAAACAGAGGTCACAGACAGATAAC	6833–8094
JEV-8R	TGGGCTCTGAAGGTTTGTAA	
JEV-9F	CAGAAGTGAAAATAACATAGTGGGAGG	7805–9005
JEV-9R	GTTTTCCCTCTCTTCATCAACCATC	
JEV-10F	GGACAGCAAAGAGTTTTCAAGGA	8730–9986
JEV-10R	TAGGAGTACCCACATTTGTGCATAT	
JEV-11F	ACTGTGCCGTCAAACCG	9682–10976
JEV-11R	AGATCCTGTGTTCTTCCTCAC	

Primers were designed according to Japanese encephalitis virus SA14-14-2 (GenBank accession number: AF315119)

[a]F and R indicate forward and reverse primers, respectively

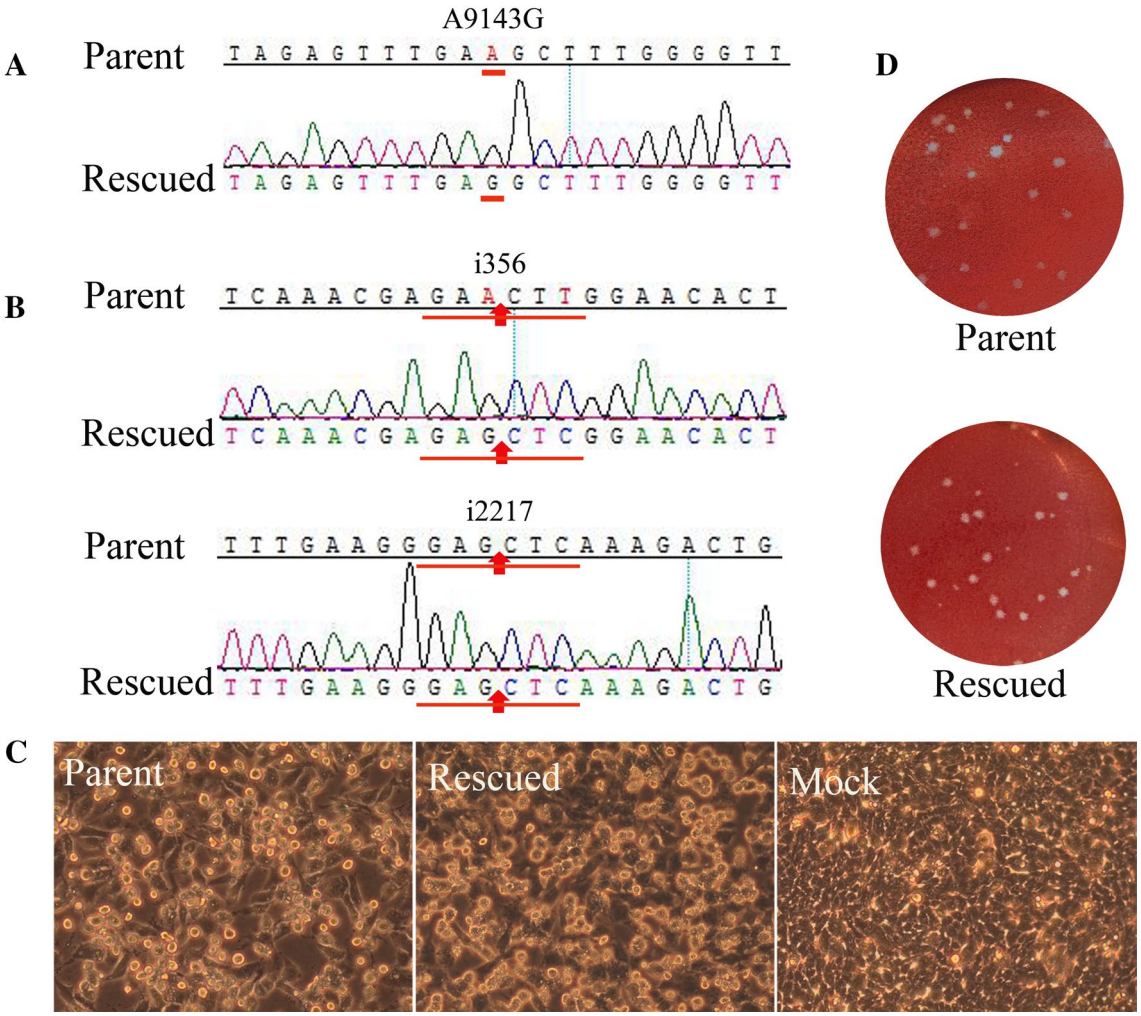

Fig. 2 Molecular marker, cytopathic effects, and plaques. **a** Molecular marker at 9143 bp (A9143G) in the rescued virus confirmed by Sanger DNA sequencing for differentiation from the parent strain. **b** Splicing out of introns at 356 bp and 2217 bp confirmed by sequencing fragments F1 and F2. Red arrows indicate sites from which introns were deleted. **c** BSR T7/5 cells infected with the parent strain and rescued strain showed a cytopathic effect 48 h after infection at an MOI of 0.25; **d** Cells were infected with 10-fold serially diluted virus and overlaid with nutrient agarose on cells. Plaques appeared 3–5 days post infection and were dyed with neutral red

10-fold serially diluted viruses were inoculated into BHK cells for incubation for 1 h at 37 °C. Subsequently, the cells were washed and incubated with DMEM containing 1.5% low melting point agarose (SeaPlaque™ Agarose, Lonza, Switzerland) and 5% fetal bovine serum (FBS). The plaques were observed 3–5 days post infection. The results indicated that both the parent and rescued virus showed a similar size and a round shape (Fig. 2d).

The expression of envelope protein was detected by an IFA protocol and there was no significant difference between the parent virus and the rescued virus (Fig. 3a). To compare the growth curve of the rescued virus with that of the parent strain, BSR T7/5 and BHK cells were both infected with the parent and rescued viruses at an MOI of 0.1. The viruses were harvested at 12 h intervals, and the growth curve was repeated three times with suspensions (culture supernatants and infected cells) titration carried in triplicate. There were no discernible differences in the growth curves between the two strains (Fig. 3b). The viral RNA copies of both strains extracted from the suspensions (culture supernatants and infected BSR T7/5 cells) 36 h post infection at an MOI of 0.1 were measured in triplicate by qRT-PCR [22]. There were an average of 18 copies/$TCID_{50}$ in the parent strain, and an average of 20 copies/$TCID_{50}$ in the rescued virus. It indicated that the infectivity of both strains is similar.

To further confirm the rescued virus, the rescued and parent strains were observed under a transmission electron microscope (TEM). BHK cells were infected with the rescued and parent virus at an MOI of 0.1. The cells were harvested and centrifuged to obtain pellets 36 h post infection. Then, the pellets were processed according to the ultrathin sectioning protocol [23] for the TEM observation. The

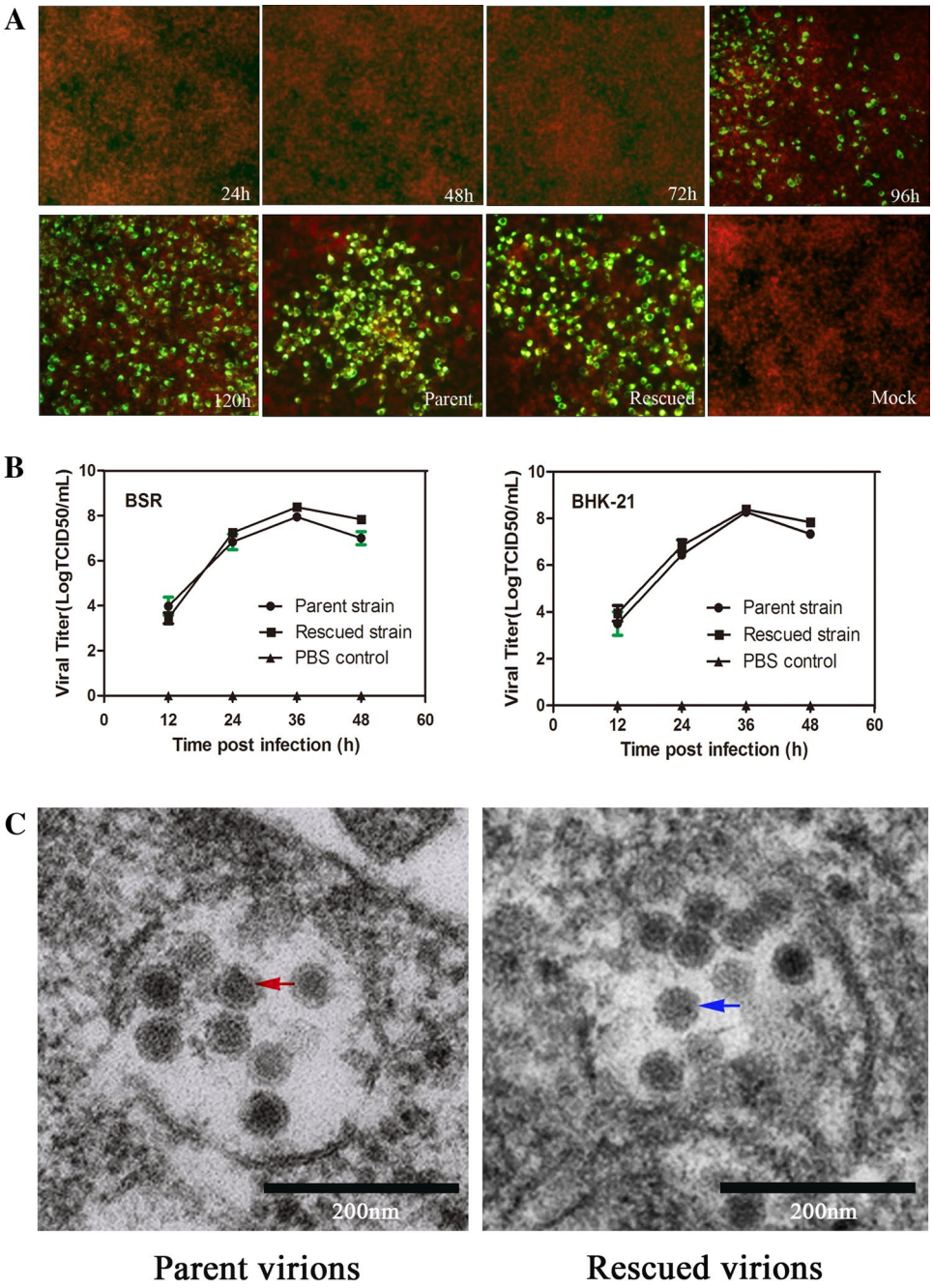

Fig. 3 IFA of viral protein expression, viral growth kinetics and morphology. **a** The rescued supernatants (collected at 24 h, 48 h, 72 h, 96 h, and 120 h post transfection) were inoculated into the BSR T7/5 cells and cultured 48 h for IFA. The BSR T7/5 cells were inoculated the parent virus, the rescued virus, and the mock at an MOI of 0.01 and were incubated 48 h for IFA. IFA was performed using a mouse-anti-JEV envelope monoclonal antibody (Abcam, Cambridge, MA, USA) as the primary antibody. Goat-anti-mouse IgG labeled with fluorescein isothiocyanate (FITC) (Thermo Scientific, Waltham, MA, USA) was used as the secondary antibody. Uninfected cells were stained with Evans blue, which showed red background by blue light at 450–480 nm. **b** The growth kinetics of parental and rescued JEV SA14-14-2 on BSR T7/5 and BHK cells showed similar characteristics at an MOI of 0.1. The growth curve and the titers were repeated 3 times. **c** Ultrathin sectioning for TEM observation was performed in cells infected with the parent ($10^{7.5}$ $TCID_{50}$/mL) and rescued ($10^{7.5}$ $TCID_{50}$/mL) viruses 36 h post infection at an MOI of 0.1 for higher viral load. Red arrows indicate the parent virions, and blue arrows indicate the rescued virions

results indicated that both viruses had similar shapes (round) and sizes (30–40 nm) (Fig. 3c). These results demonstrated that the biological properties of the rescued virus resembled those of the parent virus.

In conclusion, we provided a simple method for the recovery of JEV. To simplify the transfection procedure, BSR T7/5 cells were used with a construction containing a T7 promoter preceding the 5′ UTR and the HDVr-T7 terminator sequence following the 3′ UTR. The rescued virus had characteristics indistinguishable from those of the parent strain. The JEV SA14-14-2 reverse genetic system could be a powerful tool for applied research and fundamental research, such as the insertion or replacement of foreign genes, point mutation or deletion of genes for vaccine screening and pathogenesis research.

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Author contributions XX, HW, YG, SY, and BZ conceived and designed the experiments. GL, HJ, XN, YZ, NF, ZC, ST, WG, FY,

LL, YZ, ZC, and NL performed the experiments and provided experiments materials or ideas.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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