Recovery of a chemically synthesized Japanese encephalitis virus reveals two critical adaptive mutations in NS2B and NS4A

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A full-length genome infectious clone is a powerful tool for functional assays in virology. In this study, using a chemical synthesized complete genome of Japanese encephalitis virus (JEV) strain SA14 (GenBank accession no. U14163), we constructed a full-length genomic cDNA clone of JEV. The recovered virus from the cDNA clone replicated poorly in baby hamster kidney (BHK-21) cells and in suckling mice brain. Following serial passage in BHK-21 cells, adaptive mutations within the NS2B and NS4A proteins were recovered in the passaged viruses leading to viruses with a large-plaque phenotype. Mutagenesis analysis, using a genome-length RNA and a replicon of JEV, demonstrated that the adaptive mutations restored replication to different degrees, and the restoration efficiencies were in the order: NS2B-T102M, NS4A-R79K, NS2B-T102M+NS4A-R79K. An in vivo virulence assay in mice showed that the recombinant virus containing double mutations showed similar virulence to the WT SA14 (GenBank accession no. M55506). This study reports the first chemically synthesized JEV. A reverse genetics assay demonstrated that substitutions of NS2B-T102M and NS4A-R79K altered JEV replication.

INTRODUCTION

Japanese encephalitis virus (JEV) belongs to the genus Flavivirus in the family Flaviviridae, which includes many other important human pathogens such as yellow fever virus (YFV), West Nile virus (WNV), tick-borne encephalitis virus (TBEV) and dengue virus (DENV). JEV is transmitted primarily by Culex mosquitoes (Rice, 2007) and is widely endemic in South and South-east Asia. Approximately 67 900 Japanese encephalitis cases typically occur annually (Campbell et al., 2011), and half of the patients recovering from infection have severe neuropsychiatric sequelae (Basumatary et al., 2013). JEV contains a positive-sense ssRNA genome of about 11 000 nt encoding a single ORF flanked by two UTRs at the 5’ and 3’ ends. Genomic RNA is translated into a large precursor, which is cleaved by host and viral proteases into three structural (capsid, precursor membrane/membrane and envelope) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins (Sampath & Padmanabhan, 2009). The 5’ and 3’ UTRs can form highly conserved secondary structures that are implicated in virus replication, translation and packaging of the genome (Brinton & Dispoto, 1988; Brinton et al., 1986; Shi et al., 1996). The structural proteins form the viral particle, whilst the non-structural proteins play critical roles in RNA replication,

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One supplementary figure is available with the online version of this paper.
virus assembly and evasion of innate immune responses (Avirutnan et al., 2006; Lindenbach & Rice, 1997, 1999; Ye et al., 2012).

Currently, there is no antiviral therapy for treatment of JEV infection, and vaccination is the most effective way to control the disease. SA14-14-2 has been licensed for use as a live vaccine in China, Nepal, India and Sri Lanka, and South Korea (Kollaritsch et al., 2009), and is a live-attenuated virus derived from its virulent parental virus, SA14 (Song et al., 2012). As a live-attenuated vaccine, the theoretical risk that SA14-14-2 virus may revert back to its virulent form exists. Studies of the molecular mechanism of virus replication and pathogenesis will help to identify novel therapeutics and to develop safer vaccines of JEV. Comparison of nucleotide sequences of virus strains with different degrees of virulence is a common approach to identify potential molecular determinants responsible for viral virulence (Ni & Barrett, 1996; Ni et al., 1994; Tajima et al., 2010; Yamaguchi et al., 2011; Zhao et al., 2005). However, the nucleotide sequences of SA14, and even for the vaccine strain SA14-14-2, published by different groups are not identical due to different passage history and cell culture conditions (Ni & Barrett, 1996; Yu, 2010). Two SA14 nucleotide sequences are available in GenBank, with accession numbers U14163 and M55506. SA14/U14163 is a strain from mouse brain preparations of SA14 virus that was isolated from a pool of Culex pipiens larvae from Xi’an, China (Ni et al., 1994). SA14/M55506 is a plaque-purified virus, derived from a mouse brain preparation containing SA14 virus following three passages in primary dog kidney cell culture (Nitayaphan et al., 1990). The sequences of these two viruses differ in nucleotides in the E, NS1, NS2A, NS2B, NS4A and NS5 regions. It is necessary to construct an infectious clone of JEV SA14 with a clear genetic background in order to study viral gene function and pathogenic potential.

Chemical synthesis of viral genome is a widely used method for construction of the infectious clone, especially in the absence of a natural template. Using this method, Cello et al. (2002) first reconstructed poliovirus from known sequences. Subsequently, various viruses including the 1918 strain of influenza viruses, human endogenous retroviruses, severe acute respiratory syndrome coronavirus and WNV were also generated using a similar method (Becker et al., 2008; Lee & Bieniasz, 2007; Orlinger et al., 2010; Tumpey et al., 2005). In this study, a cDNA clone of JEV was constructed for the first time by using a chemically synthesized complete genome of strain SA14 (GenBank accession no. U14163). The recombinant viral RNA derived from the cDNA clone was not infectious, and the adaptive mutations of NS2B-T102M and NS4A-R79K were found to be essential for virus replication using a genomelength RNA and a replicon of JEV. An in vivo virulence assay in mice showed that the recombinant virus containing these double mutations showed similar virulence to the WT SA14 in our laboratory.

**RESULTS**

**Construction of a full-length JEV SA14/U14163 clone**

In this study, we chose the sequence of SA14/U14163 with a comparably simple passage history for construction of the JEV SA14 infectious clone. Four fragments covering the complete genome sequence of SA14/U14163 were synthesized by GENEWIZ (China). The low-copy-number plasmid pACYC177 and Escherichia coli HB101 were chosen as the plasmid vector and host bacterial strain, respectively, for cloning in this study. The overall scheme of the SA14/U14163 cDNA clone is outlined in Fig. 1(a). Briefly, a T7 promoter with an additional G for transcription efficiency and a hepatitis delta virus ribozyme (HDVr) sequence for the production of an authentic 3’ end of the viral RNA were inserted before the 5’ and after the 3’ end of the genome, respectively. The full-length cDNA clone pACYC-JEV-SA14/ U14163 was generated by combining the four fragments together as indicated in Fig. 1(a).

**Characterization of the full-length JEV-SA14/ U14163 clone**

SA14/U14163 recombinant genome-length RNA derived from the cDNA clone by in vitro transcription (T7 mMACHINE kit; Ambion) was electroporated into baby hamster kidney (BHK-21) cells. The transfected cells were subjected to a specific infectivity assay (SIA) and an immunofluorescence assay as described in Methods. By counting the plaque numbers, a specific infectivity value (SIV) was calculated as the number of p.f.u. (μg RNA)^{-1}. For the immunofluorescence assay (IFA), a mAb against the flavivirus envelope protein (Millipore) was used for the detection of viral protein expression in transfected cells. Unexpectedly, the transcribed RNA did not produce viable viruses efficiently in transfected BHK-21 cells, as the SIV of the RNA transcript was zero and only a small number of IFA-positive cells was observed up to 120 h post-transfection (p.t.) (Fig. 1b, c). As SA14/U14163 is a strain derived from mice brain (Ni et al., 1994), it was possible that the transcribed RNA might produce viruses efficiently in mice brain instead of in cell culture. Thus, we directly inoculated the RNA transcripts into suckling mice brain. However, no clinical signs were observed in the inoculated mice and no infectious virus was recovered from mice brains using plaque assay (data not shown).

**Revertant analysis reveals compensatory mutations within NS2B and NS4A**

As shown in Fig. 1(b), IFA-positive cells appeared at 120 h p.t., suggesting that revertant viruses might have emerged. The supernatant from cell cultures of transfected cell (designated P0 viruses) was then passaged in BHK-21 cells, and the culture supernatants from each passage were tested for replicating viruses by plaque assay. As expected, after
the supernatant had been passaged once in BHK-21 cells for 5 days (designated P1 viruses for first passage), infectious virus was recovered as shown by the appearance of small plaques (Fig. 1d). Passaging was performed for seven rounds (5 days per round) until the passaged viruses exhibited a stable large-plaque phenotype (Fig. 1d). The appearance of plaques and plaque morphology changes between the P1 and P7 viruses indicated that adaptation had occurred in the recovered viruses. Complete genome sequencing of the P7 viruses revealed four nucleotide
Compensatory mutations rescue viral production of the full-length JEV-SA14/U14163 clone

To examine whether these adaptive mutations contribute to virus replication, we prepared genome-length RNAs containing a single mutation (NS2B-T102M or NS4A-R79K) or a double mutation (NS2B-T102M+NS4A-R79K) using pACYC-JEV-SA14/U14163 as a backbone. Three parameters were used to compare the replication efficiencies of these mutant RNAs. First, plaque sizes and SIVs were determined. The NS2B-T102M mutant RNA generated pin-point-sized plaques, and yielded an SIV of \( \times 10^5 \) p.f.u. (μg RNA)^−1, whereas NS4A-R79K mutant RNA generated plaques slightly larger than the NS2B-T102M plaques and the resulting SIV was \( \times 10^4 \) p.f.u. (μg RNA)^−1. The double mutant NS2B-T102M+NS4A-R79K further increased the SIV [\( \times 10^5 \) p.f.u. (μg RNA)^−1] and plaque size (Fig. 2a). Secondly, IFA was performed to monitor viral protein expression in cells transfected with various RNAs. All mutant RNAs produced IFA-positive cells at 24 h p.t., and with increasing time, the number of IFA-positive cells also increased. The numbers of IFA-positive cells at each time point were in the order: NS2B–NS3pro to facilitate the detection of protein expression

<table>
<thead>
<tr>
<th>GenBank accession no.</th>
<th>Strain</th>
<th>Genotype</th>
<th>Nucleotide sequence of viral genomic RNA</th>
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<tr>
<td></td>
<td></td>
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<td>NS2B (nt 4511–4530)</td>
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<td></td>
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<td>NS4A (nt 6692–6710)</td>
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<tr>
<td>U14163</td>
<td>SA14</td>
<td>III</td>
<td>C C T G G C A G T C T T G C A T T G</td>
</tr>
<tr>
<td>–</td>
<td>rSA14</td>
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<tr>
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To measure directly the effect of these mutations on viral RNA replication, a transient replicon system of JEV was used. The replicon was constructed using pACYC-JEV-SA14/U14163 as a backbone by fusion PCR as described in Methods (Fig. 3a, SA14/U14163-Replicon). Consistent with the results derived from the genome-length RNAs, the luciferase signals at \( \geq 48 \) h p.t. showed differences in replication efficiencies among the various replicons in the following order: SA14/U14163<NS2B-T102M<NS4A-R79K<NS2B-T102M+NS4A-R79K (Fig. 3b). These results provided further evidence that the two site mutations, NS2B-T102M and NS4A-R79K, especially the R79K mutation in NS4A, are critical for restoring virus replication.

Thr-to-Met substitution at position 102 in NS2B has no effect on NS3 protease activity

NS2B mainly acts as an essential co-factor of NS3 protease (Chambers et al., 1991; Evans & Grant, 1977; Jan et al., 1995). To examine whether the ability of the NS2B-T102M mutant to rescue viral RNA replication correlated with the restoration of its co-factor function in the NS2B/NS3pro protease complex, we prepared constructs to express polyproteins of NS2B–NS3pro with either Thr or Met at position 102 in NS2B. The recombinant proteins contained full-length NS2B linked to the N-terminal protease domain (aa 1–180) of NS3 (Clum et al., 1997; Junaid et al., 2012; Shiryaev et al., 2006), and an haemagglutinin (HA) tag was engineered to the C terminus of NS2B–NS3pro to facilitate the detection of protein expression.
and cleavage activity (Fig. 4a). Polyprotein NS2B–NS3\textsuperscript{pro} and the auto-cleavage product of NS3\textsuperscript{pro} would be detected by the anti-HA antibody. Another two constructs of NS2B–NS3\textsuperscript{pro} with a mutation in the NS3 protease catalytic site (NS3-D75A) (Ryan et al., 1998) or a co-factor mutation of NS2B (DDDG-80–83-AAAA) (Radichev et al., 2008) were produced as negative controls. Equal amounts of different NS2B–NS3\textsuperscript{pro} mutants were transfected into 293T cells and cell lysates were subjected to Western blotting to test for protease activity. As shown in Fig. 4(b), no cleavage products were detected in the NS3 protease mutant and N2B co-factor mutant. Similar amounts of NS3\textsuperscript{pro} auto-cleavage product were observed in NS2B-102T- and NS2B-102M-transfected cells, which indicated a similar proteinase activity for both constructs.

Recombinant SA14 virus (rSA14) containing a double mutation (NS2B-T102M+NS4A-R79K) is as virulent as WT SA14

The virulence of rSA14 with the NS2B-T102M+NS4A-R79K mutations was investigated in mice. WT SA14 virus (GenBank accession no. M55506) in our laboratory has been demonstrated to be highly virulent in mice (Ye et al., 2012). As shown in Fig. 5, SA14 or rSA14 caused a dose-dependent mortality in mice. Typical neurological manifestation was also observed in mice inoculated with either virus. There was no significant difference in LD\textsubscript{50} between SA14 (LD\textsubscript{50}=10\textsuperscript{5.92} p.f.u.) and rSA14 (LD\textsubscript{50}=10\textsuperscript{6.41} p.f.u.). In addition, to test the \textit{in vivo} stability of the NS2B-T102M and NS4A-R79K mutations, brains from moribund rSA14- and WT SA14-infected mice were harvested and viral RNA was extracted for sequencing. The results showed that the viruses isolated from these two mice groups still retained both the NS2B-102M and NS4A-79K residues (Fig. S1, available in the online Supplementary Material). Overall, these results demonstrated that the recovered rSA14 virus with the NS2B-T102M+NS4A-R79K mutations showed similar pathogenicity in adult mice to the WT SA14 and could be used for future \textit{in vivo} assays.

**DISCUSSION**

In this study, we successfully constructed, for the first time, a full-length cDNA clone of JEV using a chemical synthesis approach. Both biochemical and reverse genetics studies revealed that aa 102 of NS2B and aa 79 of NS4A, especially the latter, play critical roles in virus replication. SA14 is a parental virus of the vaccine strain SA14-14-2. Construction of a full-length cDNA clone of SA14 with a clear genetic background is necessary for studying viral gene function and pathogenic potential. Using the known genome sequence of SA14/U14163, we assembled four chemically synthesized fragments that covered the whole genome and generated a full-length cDNA clone of SA14/U14163 (Fig. 1a). However, the IFA and SIA results showed that this cDNA clone could not replicate and infect efficiently (Fig. 1b, c). Sequence alignment of different JEV
strains showed that SA14/U14163 contained two unique amino acid changes at NS2B-102T and NS4A-79R that were different from the other JEV virus strains, with NS2B-102M and NS4A-79K (Table 1). This is consistent with the findings of Ni et al. (1995) when they compared amino acid sequences between SA14/U14163 and another panel of JEV strains. Interestingly, there were just two compensatory mutations, NS2B-T102M and NS4A-R79K, that occurred in the recovered viruses (Fig. 1d, e). A single mutation (NS2B-T102M or NS4A-R79K) and a double mutation (NS2B-T102M + NS4A-R79K) led to an improvement in virus replication ability to different degrees (Figs 2 and 3). An in vivo mouse assay also demonstrated that the recombinant virus with NS2B-T102M and NS4A-R79K could cause a virulent phenotype similar to that of the WT SA14 virus, which has been demonstrated to be highly virulent (Fig. 5). In addition, these two sites were stable in the infected mice (Fig. S1).

NS2B is generally thought to exert its functions in virus replication mainly through serving as a co-factor of NS3 protease (Chambers et al., 1991; Evans & Grant, 1977; Jan et al., 1995). NS2B and NS3 protease can form a

![Diagram](http://vir.sgmjournals.org)

**Fig. 3.** Analysis of adaptive mutations in replicon system. (a) Schematic of the construction of SA14/U14163-Replicon. The cDNA clone of pACYC-JEV-SA14/U14163 as a template was used as backbone for the construction of SA14/U14163 replicon (Rluc-rep). Fusion PCRs were performed to replace structural genes with the Rluc 2A gene and the final fusion PCR product was engineered at KpnI and BspEI sites of pACYC-JEV-SA14/U14163. (b) Rluc-rep was used for direct measurement of the effect of the NS2B-T102M and NS4A-R79K mutations on viral RNA replication. Equal amounts of different Rluc-rep RNAs were transfected into BHK-21 cells and assayed for luciferase activity at the indicated time points p.t.
hydrophobic regions of NS2B mainly play a role in anchoring the NS2B–NS3 complex into the host endoplasmic reticulum (ER) membrane and efficient activation of the NS3 protease domain (Clum et al., 1997). Using an in vitro system, we found that the Thr-to-Met substitution at position 102 in NS2B had no effect on the NS3 protease. In contrast, the NS3 protease mutant or the NS2B co-factor mutant lost NS3 protease activity (Fig. 4). Similar results were also found in YFV in which the deletion of aa 100–102 combined with a point substitution at aa 99 in NS2B was lethal for YFV replication, although these mutations only led to a slight reduction in proteolytic processing (Chambers et al., 1993). Therefore, the restoration of virus replication conferred by NS2B-T102M may involve an unknown role of NS2B in virus replication other than as a co-factor of the NS3 protease. Recently, Yu et al. (2013) demonstrated an interaction between NS4A and NS2B by in vitro imaging assays. As addition of NS2B-T102M to NS4A-R79K exhibited an additive effect, it is possible that there is a genetic or/and physical interaction between NS2B and NS4A in virus replication.

NS4A has been shown to be involved in the formation of the virus replication complex (Miller et al., 2007) and immune evasion (Ambrose & Mackenzie, 2011; Liu et al., 2005), as well as the induction of autophagy (McLean et al., 2011). This protein consists of an N-terminal cytosolic domain (aa 1–50), a region that is closely associated with the ER luminal side of the lipid bilayer (pTMS2 region; aa 74–100), and three transmembrane regions (Miller et al., 2007). Apparently, residue 79 in EVV NS4A localizes in the pTMS2 region. It was supposed that insertion of the pTMS2 region into the luminal leaflet of the ER membrane might act like a wedge, resulting in NS4A-induced membrane curvature, facilitating virus replication (Miller et al., 2007). Flaviviruses always prefer a positively charged amino acid – Arg (YFV and TBEV) or Lys (JEV, DENV and WNV) – at this position. These positively charged residues in membrane proteins are often believed to interact with a negatively charged phospholipid head group, assisting in anchoring the transmembrane orientation (de Planque et al., 1999; Gleason et al., 2013). Lys is more flexible and is more prone to extend to the surface with its amino group exposed compared with Arg (Baud & Karlin, 1999).

Our results showed that a single Arg-to-Lys substitution in NS4A completely altered SA14/U14163 replication ability. It remains to be determined whether this substitution changes the topology structure of NS4A by affecting the anchor of the pTMS2 region in the ER membrane.

**METHODS**

**Cells and antibodies.** BHK-21 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹ in 5% CO₂ at 37 °C. A mAb against the E protein of St Louis encephalitis virus (SLEV) was purchased from Chemicon, and is cross-reactive with the JEV E protein. Texas Red-conjugated goat anti-mouse IgG was purchased from ProteinTech Group.

**Plasmid construction.** A complete genome-length cDNA clone of JEV-SA14/U14163 was constructed by chemical synthesis. Briefly,
the full-length genome sequence was divided into four fragments (Fig. 1a) and artificially synthesized by the DNA synthesis company GENEWIZ. Fragment A contained a T7 promoter proceeding sequence from the 5’ end to nt 3 (450 nt), and fragment B contained nt 3446–5581 of the genome. Both fragments were cloned into the low-copy-number vector pACYC177 at the PstI and BamHI sites. Fragment C containing nt 5576–9136 and fragment D containing nt 9121–10976 of the genome with a HDVr sequence were cloned into pACYC177 at the BamHI and XhoI sites. Next, fragment A + B was constructed by pasting fragment B into fragment A at the BspEI (nt 3446) and BamHI (nt 5577) sites. Fragment C + D was generated by engineering fragment D into fragment C at the BamHI and XhoI sites. Construction of pACYC-JEV-SA14/U14163, which contained a genome-length cDNA of JEV-SA14/U14163, was obtained by inserting fragment C + D into fragment A + B at the BamHI and XhoI sites.

A Renilla luciferase (Rluc) reporting replica was constructed by replacing the structural genes with the Rluc gene in frame through two rounds of overlap PCR using pACYC-JEV-SA14/U14163 as a backbone. The fragments ‘KpnI–T7pro–5’ UTR–Capsid341 and ‘E10–NS1’ were amplified with the primer pairs indicated in Fig. 3(a) using pACYC-JEV as template, and the ‘Rluc–2A’ fragment was amplified using a WNV–Rluc–2A replicon (Lo et al., 2003) as template. The three fragments were fused together to produce the fragment ‘KpnI–T7pro–5’ UTR–Capsid341–Rluc–2A–E10–NS1’ by two-step overlap PCR as indicated in Fig. 3(a). The overlap sequences in all three fragments are indicated with grey shaded boxes. The resulted PCR products were cloned into the low-copy-number vector pACYC177 at the XhoI and NotI sites.

RNA transcription and RNA electroporation transfection. The infectious clone and replica cDNA plasmids were digested with XhoI and purified using phenol/chloroform extraction. The linearized cDNAs were then subjected to in vitro transcription using a mMEGAscript T7 kit (Ambion). All procedures were performed according to the manufacturer’s protocols. The RNA was resolved in RNase-free water and stored at −80 °C in aliquots. Approximately 5 μg RNA was electroporated into 8 × 10⁶ BHK-21 cells in 0.8 ml ice-cold PBS buffer (pH 7.5) in a 0.4 cm cuvette with a GenePulser apparatus (Bio-Rad) at 850 V and 25 μF, pulsing three times at 3 s intervals. After a 10 min recovery at room temperature, the transfected cells were mixed with 25 ml pre-warmed DMEM containing 10% FBS.

SIA. For SIA, approximately 5 × 10⁵ BHK-21 cells were seeded into six-well plates the day before RNA electroporation. A series of 1:10 dilutions was made by mixing 1 ml RNA-transfected cell suspension (described above) with 9 ml DMEM containing 10% FBS. One millilitre of each dilution was added to each well of the six-well plates containing BHK-21 monolayers. After 6 h incubation at 37 °C, the culture medium was aspirated and the cells were covered with a first layer of agar. A second layer of agar containing neutral red was added after 72 h of incubation at 37 °C with 5% CO₂. The number of plaques was counted after an additional 12–24 h of incubation. The SIV was calculated as the number of p.f.u. (μg transfected RNA)⁻¹.

IFA. The RNA-transfected cells were seeded on a Chamber Slide (Nalge Nunc). At 24, 48 and 72 h p.t., the cells were fixed in cold (−20 °C) 5% acetic acid in methanol for 10 min at room temperature. The fixed cells were washed with PBS three times and then incubated with anti-SLEV E protein mAb (1:250 dilution in PBS) for 1 h. After washing with PBS three times, the cells were incubated with goat anti-mouse IgG conjugated to Texas Red at room temperature for another hour. After three PBS washes, the slide was mounted with 95% glycerol and examined under a fluorescence microscope. Cell images were taken at ×200 magnification.

Plaque assay. Virus stock was produced by harvesting the supernatant of RNA-transfected BHK-21 cells. Virus titre and morphology were determined by plaque assay. Briefly, a series of 1:10 dilutions were prepared by diluting 15 μl virus stock with 135 μl DMEM containing 10% FBS, and 100 μl each dilution was seeded onto each well of a six-well plate containing confluent BHK-21 cells (5 × 10⁵ cells per well, plated 1 day in advance). The plates were incubated at 37 °C with 5% CO₂ for 1 h before the first layer of agar was added. After 72 h of incubation at 37 °C with 5% CO₂, a second layer of agar containing neutral red was added. Plaques were photographed and the number determined after neutral red incubation of the plates for another 12–24 h. The viral titre was calculated as p.f.u. ml⁻¹. The limit of detection was 10 p.f.u. ml⁻¹.

Luciferase assay. Replicon RNA-transfected cells were seeded in 12-well plates in various amounts. Briefly, 1, 1, 0.5, 0.25 and 0.25 ml transfected cells was seeded and the cell lysates were recloned at 4, 24, 48, 60 and 72 h p.t. and stored at −80 °C for subsequent luciferase assay. Triplicate wells were seeded for each time point. Luciferase activity was measured in a Microplate Reader (Varioskan Flash; Thermo Fisher) by mixing 20 μl lysis with 50 μl substrate (Promega). The limit of detection was 100 light units.

Western blotting. One microgram of various pCAGGS-based NS2B–NS3-expressing plasmids was transfected into BHK-21 cells in a 3.5 cm dish using Lipofectamine 2000. At 48 h p.t., the cells lysates were collected by incubation with 200 μl lysis buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris/HC1 (pH 7.5), 1 mM EDTA and 1 mM PMSF for 20 min on ice. After centrifugation at 5500 g for 5 min, the samples were analysed by SDS-PAGE (12% acrylamide) and transferred onto PVDF membranes (Millipore), followed by blocking with 5% non-fat milk in TBS-T (24 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20 for 1 h. After sequential incubation with primary anti-HA mAb (Pierce) and secondary anti-mouse antibody conjugated to HRP (Bio-Rad), the signals were visualized with a chemiluminescence system (ChemilDoc; Bio-Rad).

Animal studies. Groups of 3-week-old female BALB/c mice (n = 6–8) were injected intraperitoneally with SA14 or rSA14 virus with serial fivefold dilutions containing 5 × 10⁶ to 2 × 10⁹ p.f.u. Mortality and any clinical symptoms were then monitored daily for 15 days. The LD₅₀ was calculated as described previously (Tan et al., 2013). All animal experimental procedures were approved by and carried out in accordance with the guidelines of the Animal Experiment Committee of the State Key Laboratory of Pathogen and Biorsecurity, China.

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