Genetic and Phenotypic Properties of Vero Cell-Adapted Japanese Encephalitis Virus SA14-14-2 Vaccine Strain Variants and a Recombinant Clone, which Demonstrates Attenuation and Immunogenicity in Mice

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Abstract. The live-attenuated Japanese encephalitis virus (JEV) SA14-14-2 vaccine, produced in primary hamster kidney cells, is safe and effective. Past attempts to adapt this virus to replicate in cells that are more favorable for vaccine production resulted in mutations that significantly reduced immunogenicity. In this study, 10 genetically distinct Vero cell-adapted JEV SA14-14-2 variants were isolated and a recombinant wild-type JEV clone, modified to contain the JEV SA14-14-2 polypeptide amino acid sequence, was recovered in Vero cells. A single capsid protein mutation (S66L) was important for Vero cell-adaptation. Mutations were also identified that modulated virus sensitivity to type I interferon-stimulation in Vero cells. A subset of JEV SA14-14-2 variants and the recombinant clone were evaluated in vivo and exhibited levels of attenuation that varied significantly in suckling mice, but were avirulent and highly immunogenic in weanling mice and are promising candidates for the development of a second-generation, recombinant vaccine.

INTRODUCTION

Japanese encephalitis virus (JEV), a member of the genus flavivirus, is maintained in a zoonotic cycle between Culex mosquitoes and ardeid birds or domestic swine and is responsible for significant epidemics of viral encephalitis in Asia.1–3 Three billion people live in regions with endemic JEV transmission resulting in an estimated 60,000 annual cases, of which 20–40% are fatal and 45–70% of survivors have neurologic sequelae.4,5 The JEV has a single-strand, plus-sense, RNA genome of ~11 kb in length, which contains a single open reading frame (ORF) that is flanked by 5¢ and 3¢ untranslated regions (UTRs).6 The single ORF codes for a polyprotein that is processed by viral and host cellular proteases into the capsid (C), precursor-membrane (prM), and envelope (E) structural proteins and non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.6 Humoral immunity is an essential component of protection from flavivirus disease and the E protein is the primary target of virus neutralizing antibodies.7–9 Innate-immune antiviral mechanisms like those induced by type I interferon (IFN) are critical for controlling virus dissemination within the host,10 whereas adaptive immune cell-mediated responses are important for virus clearance.11–13

Inactivated JEV vaccines have been available since the mid-1950s and have been important for controlling the incidence of disease caused by JEV.14–16 However, their high production costs and requirement for multiple doses make inactivated vaccines less practical for mass vaccination campaigns in most rural JEV-endemic areas. The live-attenuated JEV SA14-14-2 vaccine (Chengdu Institute of Biological Products, China) was licensed in China in 1988 and has more recently become available for use in Cambodia, India, Korea, Laos, Myanmar, Nepal, Sri Lanka, and Thailand.15,17,18 Immunization with JEV SA14-14-2 has been shown to result in 88–96% efficacy after a single dose (105.4–106.8 plaque-forming units [PFU]/dose, depending on the study19–25) and has been administered to millions of children with no reported serious adverse events.15,23–26 The World Health Organization (WHO) prequalified the JEV SA14-14-2 vaccine in October of 2013.

The SA14-14-2 vaccine strain was derived from wild-type (WT) JEV strain SA14, which was originally isolated from mosquito larvae by numerous passages in mouse brain. The JEV SA14 underwent 100 passages in primary hamster kidney (PHK) cells, several plaque purifications in primary chick embryo cells, peripheral passages in Syrian hamsters, and suckling mice, followed by additional plaque purifications in PHK cells, which resulted in the accumulation of attenuating mutations found in the JEV SA14-14-2 genome.27–30 There appear to be multiple attenuating mutations in the JEV SA14-14-2 E protein,31 however E-E138K seems to be particularly important.32–35 Interestingly, a recombinant WT JEV Nakayama virus bearing the 5¢ UTR, C, prM, and E genes of JEV SA14-14-2 had residual neurovirulence in mice, indicating that mutations located outside the structural protein genes might also contribute to attenuation.36 More recently it was shown that a single nucleotide change in the JEV SA14-14-2 NS2A coding region that ablates NS1’ formation contributes to the attenuation phenotype.37 Furthermore, JEV SA14-14-2 appears to be more sensitive to type I IFN in tissue culture than some WT JEV strains,38 which might be a result of less efficient type I IFN signaling antagonism by its NS5 protein,39 and this probably contributes to its attenuation as well. However, the complete set of mutations that contribute to balanced attenuation and immunogenicity of JEV SA14-14-2 have not been fully elucidated.

International use of the JEV SA14-14-2 vaccine was limited, for many years, by safety concerns related to its production in PHK cells, which require maintenance of pathogen-free hamster colonies as a source of kidneys for cell culture in medium containing bovine serum.18 The continuous Vero cell line, originally derived from African green monkey kidney, offers several advantages over PHK cells. A qualified WHO Vero cell bank was established for the production of biological products40 and methods for large-scale vaccine production are well established.41,42 Furthermore, Vero cells are highly permissive to viral infection,43 which is likely caused by a defect in type I IFN production.44 Previous attempts have been made...
to adapt JEV SA14-14-2 to more suitable cell substrates and have resulted in mutations in the virus that reduced vaccine immunogenicity. In the current study, several genetically distinct Vero cell-adapted JEV SA14-14-2 variants were isolated and a recombinant clone bearing the polyprotein amino acid sequence of JEV SA14-14-2 was constructed and recovered in Vero cells. The in vitro and in vivo phenotypes of these viruses were compared and suggest that Vero cells are a suitable substrate for the development of a safe and immunogenic, second-generation, live-attenuated JEV SA14-14-2 vaccine.

MATERIALS AND METHODS

**Cells.** The WHO Vero (African green monkey kidney) cells were maintained at 37°C in OptiPRO SFM (serum-free medium) (Gibco) supplemented with 4 mM L-glutamine (Gibco). Raji (human B-cell lymphoblast) cells were maintained at 37°C in RPMI 1640 medium (Gibco) supplemented with 10% FBS (HyClone, GE Healthcare Life Sciences, Logan, UT), 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin (Gibco). The HEK-293T (human kidney) cells were maintained at 37°C in EMEM (Lonza, Basel, Switzerland) supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin-streptomycin.

**Viruses.** The JEV genotype I strain M859 (Cambodia/67), genotype II strain B1065 (Thailand/83), genotype III strain 7812474 (India/78), and genotype IV strain JKT7003 (Indonesia/Java/81) were obtained from the World Reference Center for Emerging Viruses and Arboviruses (University of Texas Medical Branch (UTMB), Galveston, TX). Virus stocks were passed once in Vero cells to generate working stocks. The recombinant (r) JEV strain (rJEV India/78) was derived from a complementary DNA (cDNA) clone that has been described previously. The JEV SA14-14-2 strain was obtained from laboratory archives and was passed once in Vero cells to obtain a working stock that was sequenced.

**Generation of Vero-adapted JEV SA14-14-2 variants.** Vero cell-adapted JEV SA14-14-2 variants were isolated by performing at least two rounds of terminal endpoint dilutions of the JEV SA14-14-2 laboratory stock in Vero cells. These biological clones underwent two additional rounds of amplification in Vero cells to generate working stocks that were sequenced. The JEV SA14-14-2 stock also underwent multiple passages in Vero cells stimulated with 10 U/mL of human IFN-α A/D (Sigma-Aldrich St. Louis, MO, catalog no. I4401) that is active on Vero cell monolayers. This was followed by biological cloning and amplification in Vero cells, in the absence of IFN-treatment, and sequencing.

**Construction of rJEVmut14-14-2.** The cDNA clone of WT rJEV India/78, which has been described previously, was modified to closely match the polyprotein amino acid sequence of the JEV SA14-14-2 GenBank reference strain (accession no. JN604986) and was designated pJEVmut14-14-2. Synthesized cDNA gene segments encoding point mutations were cloned into pJEV India/78 (Figure 1). The T39A (5' UTR) and T292C (C-L66S) mutations were cloned as an AscI-BsrWI cDNA fragment. Mutations G4403T/A4408G (NS2B-E63D/D65G) and A4782G/C4921G/T4922C (NS3-M59V/A105G) were cloned as an FseI-Stul fragment. Mutation A7227G (NS4B-I106V) was cloned as a BglII-MluI fragment. Mutations T9688C (NS5-V671A) and T10428C (3' UTR) were cloned as a PmlI-Acc65I fragment. The remaining portions of the recombinant clone were derived from polymerase chain reaction (PCR) amplified cDNA segments of JEV SA14-14-2 (Figure 1). Nucleotides 625–2496, which include a large portion of prM and the entire E gene, were cloned as a BsiWI-PvuI cDNA fragment. Nucleotides 2491–4226, which include the NS1 and NS2A genes, were cloned as a PvuIFseI cDNA fragment. A portion of the NS5 gene (nucleotides 8162–8952) was cloned as a MluI-PvuII cDNA fragment. To improve stability of the pJEVmut14-14-2 cDNA construct in *Escherichia coli*, a DNA linker containing multiple stop codons was synthesized with forward sequence 5'–CGTGACTTAAA CTTAGACTACGAT-3' and reverse sequence 5'–CGTATCT AAATGTTAAGTCACGAT-3'. The DNA oligo nucleotides were annealed in vitro and ligated into the PvuI restriction site.

**Recovery of rJEVmut14-14-2.** Recovery of rJEVmut14-14-2 was accomplished by first removing the PvuI stop codon linker sequence with a PvuI digest, followed by in vitro ligation to recircularize the cDNA plasmid. The cDNA construct was subsequently linearized with Acc65I and full genome-length capped RNA transcripts were synthesized with the AmpliCap SP6 Message Maker Kit (Epigencentre Biotechnologies, Madison, WI). Purified transcripts were transfected in Vero cells using DOTAP liposomal transfection reagent (Roche Basel, Switzerland). Transfected cell culture supernatant was harvested 3 days later and titered in Vero cells. Recovered virus underwent multiple terminal dilutions, followed by amplification in Vero cells to generate a biological clone, which was sequenced.

**Virus replication in Vero cells.** Vero cells were infected with viruses at a multiplicity of infection (MOI) of 0.01 PFU/cell for 1 hr, and then washed twice with phosphate buffered saline (PBS) (Gibco) followed by addition of media. The media was harvested on Day 3 post-infection, which corresponded to peak virus titer, and was titered in Vero cells. Plaques were visualized on Day 3 by immunostaining and their relative size (small < 1 mm, medium 1–2 mm, and large > 2 mm) was determined. The results are an average of at least two experiments done in duplicate per virus.

**Type 1 IFN sensitivity assay.** Virus was added to Vero cells in 24-well plates at an MOI of 0.01 PFU/cell and incubated for 1 hr followed by addition of media-only or media containing IFN-α (Sigma-Aldrich St. Louis, MO, catalog no. I4401) followed by addition of media. The virus titer in the media was determined by plaque assay on Day 3 post-infection.
10 U/mL of IFN-α A/D (Sigma-Aldrich), in duplicate per experiment. Virus dilutions used for infection were immediately back titrated in Vero cells to ensure consistent doses/well. The supernatant was harvested 72 hr post-infection and titrated in Vero cells. Dilution of the supernatant > 100-fold during titering reduced any residual IFN-α to a concentration at which plaque count was not affected (data not shown). The cell monolayers were washed once with PBS, removed with TrypLE select (Life Technologies), transferred to 5 mL tubes with growth medium, and pelleted by centrifugation. Cell pellets were resuspended with FACS Permeabilizing Solution 2 (BD Biosciences, San Jose, CA) and incubated for ~1 hr at room temperature. The JEV Ag-positive cells were detected by incubating with a JEV hyperimmune mouse ascitic fluid (ATCC, Manassas, VA) diluted in PBS containing 10% albumin (wash solution) and then with a FITC-goat anti-mouse H+L antibody (Biolegend, San Diego, CA) diluted in wash solution. The cells were washed twice with PBS, resuspended in PBS with 0.01% EDTA, and data were collected using an Accuri C6 flow cytometer (BD Biosciences) and analyzed with Flowjo software version X.0.7 (Flowjo, Ashland, OR).

**Mouse studies.** Studies in mice were performed in accordance with the regulations and guidelines of the National Institutes of Health (NIH) (Bethesda, MD) and approved by the Animal Care and Use Committee of National Institute of Allergy and Infectious Diseases (NIAID). Viruses were tested for neuroinvasiveness by intraperitoneal (IP) inoculation and neurovirulence by intracerebral (IC) inoculation of 3-week-old (weanling) female Swiss Webster (SW) mice (Taconic, Derwood, MD). Groups of 10 mice were administered a 0.1 mL dose IP or a 0.01 mL dose IC of virus diluted and monitored for 21 days for signs of encephalitis. On Day 28, serum was collected from IP inoculated mice for neutralization assays. Viruses were also tested for neurovirulence by IC inoculation of 3-day-old (suckling) SW mice. Litters of 10 mice were administered a 0.01 mL dose of virus dilution and monitored for 21 days for signs of encephalitis. Clinical signs of encephalitis leading to a moribund state included tremors, seizure, and paralysis. Moribund mice were humanely euthanized to minimize pain and distress. The LD_{90} values were determined by the method of Reed and Muench.49

**Production of JEV reporter virus particles (RVPs).** A Bs/W1-MfeI cDNA fragment corresponding to the JEV SA14-14-2 E gene and a portion of prM was cloned into mammalian expression vector pJEV-CprME.57 The JEV SA14-14-2 RVPs expressing green fluorescent protein (GFP) were produced in HEK 293T cells, as described previously.47,50,51 The RVP titers were determined by serial dilution of JEV RVP stocks and infecting Raji cells in 96-well plates. The RVP-infected cells were fixed with paraformaldehyde and the percentage of GFP expressing cells was determined by flow cytometry.

**JEV RVP serum neutralization assay.** The JEV RVP serum neutralization assay was carried out as described previously.47 The data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA) and were fit by a dose–response curve (variable slope) to determine the 50% effective neutralization concentration (EC_{50}). An immunized mouse seroconverted if its serum had a detectable EC_{50}. Titers are reported as the reciprocal geometric mean EC_{50} titer.

**Statistical analysis.** Statistical analyses were done using GraphPad Prism. Virus titer or number of virus Ag-positive cells with or without IFN-α treatment was analyzed using a two-way analysis of variance (ANOVA). The Pearson correlation coefficient (r) was calculated, with P < 0.05 required for significance, to assess relationships between virus titer, number of virus Ag-positive cells, and IFN-α treatment. Mouse survival analyses were done using the Log-rank (Mantel-Cox) test, with P < 0.05 required for significance.

**RESULTS**

**Sequence comparison of JEV SA14-14-2 variants.** The consensus sequence of the JEV SA14-14-2 laboratory stock differed by a single amino acid substitution (NS3-A235V) relative to a recently published “reference sequence” (GenBank accession no. JN604986) of a commercial batch of JEV SA14-14-2 vaccine (Table 1).48 Careful examination of the sequence chromatograms revealed that there were also minor secondary peaks at several positions throughout the genome indicating there was a mixture of nucleotides and predicted amino acid substitutions (Table 1). Biological cloning of this virus stock in Vero cells resulted in the isolation of six genetically distinct, Vero cell-adapted variants (A, B, C, D, G, and H). These virus variants all contained nucleotide change C292T (S66L amino acid change) in the C protein gene, which appeared as a mixture of C and T nucleotides in the consensus sequence of the original laboratory virus stock (Table 1). Therefore, the principal virus in the JEV SA14-14-2 laboratory stock, bearing C-S66, was negatively selected during Vero cell-adaptation, which resulted in reversion to the Leu codon found in WT JEV. The sequence of all six Vero-adapted JEV SA14-14-2 variants also differed from the reference sequence by nucleotide change C5311T, resulting in mutation NS3-A235V, just like the original virus stock. The sequences of JEV SA14-14-2 variants A and G also contained four unique mutations compared with the reference sequence (Table 1). The sequences of JEV SA14-14-2 variants B, C, and D each contained a total of seven reversion mutations (>90% frequency in WT JEV strains), including C-S66L, and variants B and C each had two unique changes compared with the reference sequence (Table 1). All of the mutations in variants A and D appeared as mixtures of nucleotides and predicted amino acids in the consensus sequence of the JEV SA14-14-2 stock, suggesting that these variants had been dominant (Table 1).

The PHK cells used for propagation of vaccine lots of JEV SA14-14-2 are capable of producing type I IFN in response to viral infection,52 and this could be important for maintaining genetic and phenotypic properties of the vaccine. Vero cells, however, have a defect in type I IFN production, but are capable of responding to stimulation by these cytokines.44 Therefore, the JEV SA14-14-2 stock was also passaged in Vero cells treated with a low dose of IFN-α followed by biological cloning and a total of five virus variants were isolated (1^{IFN-α}, 2^{IFN-α}, 3^{IFN-α}, 4^{IFN-α}, and 5^{IFN-α}). These virus variants shared the C-S66L mutation and all but one had the NS3-A235V mutation (Table 1). The sequence of JEV SA14-14-2 variant 1^{IFN-α} was most similar to variant A, but contained substitution NS1-G174E, a change to the reference sequence at this position (Table 1). The JEV SA14-14-2 variant 2^{IFN-α} differed from the reference sequence and variant H by three mutations and had unique mutations in E and NS4A (Table 1). The JEV SA14-14-2 variants 3^{IFN-α}, 4^{IFN-α}, and 5^{IFN-α} shared NS3-A235V and the six reversion mutations specific to variants B, C, and D (Table 1).
The JEV SA14-14-2 variants 3IFN and 4IFN had identical sequences, and differed from variant D by reversion mutation NS2A-V40A (Table 1). The JEV SA14-14-2 variant 5IFN differed from variant D by unique substitutions in NS2B and NS3 (Table 1). Thus, Vero-adapted JEV SA14-14-2 variants isolated with IFN-α treatment were genetically distinct but representative of those isolated without treatment.

**Recovery of rJEVmut14-14-2.** The polypeptide amino acid sequence of rJEVmut14-14-2 was designed to closely match the sequence of a commercial batch of vaccine virus (Table 2). Other JEV SA14-14-2 full genome sequences that have been published are shown in Table 2 for comparison and differ considerably from one another. Recombinant JEVmut14-14-2 was recovered and biologically cloned in Vero cells and the sequence of this virus matched that of the cDNA clone. The genetic stability of this virus was determined by performing six additional passages in Vero cells, in duplicate, followed by sequencing of the virus genomes. At passage six, a single nucleotide change (C292T) was identified for both independent passages. This time-point was chosen because it accurately represented the cumulative effect of IFN-α treatment on virus replication over the course of 3 days in tissue culture. The JEV SA14-14-2 variant 3IFN had a single unique reversion mutation, NS2A-V40A, relative to JEV SA14-14-2 variant D (Table 1) that significantly increased mean peak virus titer (Table 3). The WT JEV strains produced large plaques (> 2 mm) and had the highest mean peak titers of 7.9–8.3 log_{10} PFU/mL in Vero cells (Table 3).

Increased sensitivity to type I IFN is an additional phenotype that has previously been associated with JEV SA14-14-2 and can be observed in IFN-treated tissue culture cells. Therefore, virus titer and virus Ag-positive cells were measured at 3 days post-infection in IFN-α-treated Vero cell monolayers. This time-point was chosen because it accurately represented the cumulative effect of IFN-α treatment on virus replication over the course of 3 days in tissue culture. The IFN-α treatment significantly reduced virus titer for all JEV strains tested (Figure 2A) and there was a significant, positive correlation (r = 0.9296, P < 0.0001) between virus titer with and without treatment (Figure 2B), indicating that virus replicative fitness is important for overcoming the antiviral effects of type I IFN-stimulation. Recombinant JEVmut14-14-2 had significantly less Ag-positive cells compared with other JEV strains that disseminated to the entire Vero cell monolayer (>5.5 log_{10} cells/well), however this was reduced significantly with IFN-α treatment (Figure 2C). There was also a significant, positive correlation (r = 0.9157, P < 0.0001) between the number of Ag-positive cells and virus titer, with IFN-α treatment, whereby viruses that infected more cells grew to higher titer (Figure 2D). In IFN-α treated Vero cell monolayers, WT JEV strains disseminated most effectively beyond the initial MOI (>3.4 log_{10} cells), whereas...
most JEV SA14-14-2 variants had low or intermediate levels of dissemination by comparison (Figure 2E). Interestingly, JEV SA14-14-2 variants $^{3}\text{IFN}$ and $^{5}\text{IFN}$ disseminated in IFN-α treated Vero cell monolayers to a similar extent as WT JEV strain Indonesia/Java/81 and significantly more than rJEVmut14-14-2, JEV SA14-14-2, and other variants (Figure 2C and E). By contrast, cell-to-cell spread of rJEVmut14-14-2 and JEV SA14-14-2 variants C and $^{3}\text{IFN}$ had remarkably reduced virus propagation, resulting in 1,000-fold reductions in dissemination and >3,000-fold reductions in virus titer (Figure 2F). Whereas, JEV SA14-14-2 variants $^{2}\text{IFN}$ and $^{5}\text{IFN}$ had type I IFN-sensitivity levels similar to WT JEV strain Indonesia/Java/81, indicating that passage of the original virus stock in IFN-α treated Vero cells enriched for more resistant virus variants (Figure 2F). It is difficult to ascribe IFN-sensitivity to specific mutations, however, at least one of three amino acid substitutions (E-M61, N3-S235A, and N4A-S45) that differentiated JEV SA14-14-2 variant $^{2}\text{IFN}$ from variant H and the NS2A-V40A mutation that differentiated JEV SA14-14-2 variant $^{3}\text{IFN}$ from variant D (Table 1) conferred a ≥5-fold significantly decreased level of sensitivity to IFN-α treatment (Figure 2F).

### Mouse studies

Virulence was tested in weanling mice by IP and IC routes of inoculation for rJEVmut14-14-2, JEV SA14-14-2, and several JEV SA14-14-2 variants and compared with WT rJEV India/78. Recombinant JEVmut14-14-2, JEV SA14-14-2, and variants A, B, C, D, G, H, and $^{5}\text{IFN}$ caused no mortality by either route of inoculation, similar to mock-infected animals (Table 4). By contrast, WT rJEV India/78 caused significant mortality (Table 4). Immuneautogenicity was evaluated in weanling mice that were inoculated IP. Immunization with rJEVmut14-14-2 at a 10$^5$ PFU dose resulted in 100% seroconversion and high neutralizing antibody titers (Table 4). The other viruses were tested at a 10$^4$ PFU dose. The JEV SA14-14-2 stock and all the variant viruses induced 90–100% seroconversion. The JEV SA14-14-2 variants B and D elicited the highest levels of neutralizing antibody, with variant G eliciting the lowest level. However, mean titers of neutralizing antibodies elicited by all the variants, except variant G, differed no more than 2-fold compared with JEV SA14-14-2 (Table 4).

### Neurovirulence

Neurovirulence was tested in suckling mice for a subset of viruses. Recombinant JEV India/78 was the most virulent in this model with an IC LD$_{50}$ of 0.2 PFU and mice had a median survival time of 5 days (Table 5). The IC LD$_{50}$ level for JEV SA14-14-2 variant H was similar to rJEV India/78 but mice had a significantly longer, 7 days, median survival time (Table 5 and Figure 3). The JEV SA14-14-2 and rJEVmut14-14-2-infected mice had the same median survival time as variant H, but slightly higher IC LD$_{50}$ levels (Table 5). The other JEV SA14-14-2 variants that were tested were significantly more attenuated. The JEV SA14-14-2 variant G differed from variant H by four amino acid substitutions and

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### Table 2

Comparison between published JEV SA14-14-2 reference sequence, rJEVmut14-14-2, and two additional full genome sequences of JEV SA14-14-2.

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<td>●</td>
<td>●</td>
</tr>
<tr>
<td>10950</td>
<td>3'UTR</td>
<td>G</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>C</td>
</tr>
<tr>
<td>10951</td>
<td>3'UTR</td>
<td>C</td>
<td>●</td>
<td>●</td>
<td>●</td>
<td>G</td>
</tr>
</tbody>
</table>

*GenBank accession nos. JN604986, AF315119, and D90195.

---

### Table 3

Table of Virus phenotype in Vero cells

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque size</th>
<th>Mean peak titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>rJEVmut14-14-2</td>
<td>Small</td>
<td>6.4 ± 0.0</td>
</tr>
<tr>
<td>rJEVmut14-14-2-C292T</td>
<td>Small</td>
<td>7.1 ± 0.1‡</td>
</tr>
<tr>
<td>JEV SA14-14-2</td>
<td>Small</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>JEV SA14-14-2 A</td>
<td>Small</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>JEV SA14-14-2 B</td>
<td>Small</td>
<td>7.4 ± 0.3</td>
</tr>
<tr>
<td>JEV SA14-14-2 C</td>
<td>Small</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td>JEV SA14-14-2 D</td>
<td>Small</td>
<td>7.3 ± 0.4</td>
</tr>
<tr>
<td>JEV SA14-14-2 G</td>
<td>Small</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>JEV SA14-14-2 H</td>
<td>Small</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>JEV SA14-14-2 JNF</td>
<td>Small</td>
<td>7.3 ± 0.2</td>
</tr>
<tr>
<td>JEV SA14-14-2 2 JNF</td>
<td>Small</td>
<td>7.3 ± 0.1</td>
</tr>
<tr>
<td>JEV SA14-14-2 3 JNF</td>
<td>Small</td>
<td>7.8 ± 0.2§</td>
</tr>
<tr>
<td>JEV SA14-14-2 5 JNF</td>
<td>Small</td>
<td>7.3 ± 0.1</td>
</tr>
<tr>
<td>JEV Cambodia/67 (GII)¶</td>
<td>Large</td>
<td>8.3 ± 0.1</td>
</tr>
<tr>
<td>JEV Thailand/93 (GII)¶</td>
<td>Large</td>
<td>8.0 ± 0.0</td>
</tr>
<tr>
<td>rJEV India/78 (GII)¶</td>
<td>Large</td>
<td>8.1 ± 0.2</td>
</tr>
<tr>
<td>JEV Indonesia/Java/81 (GIV)¶</td>
<td>Large</td>
<td>7.9 ± 0.0</td>
</tr>
</tbody>
</table>

*Measured 3 days post-infection: small < 1 mm, large > 2 mm.
‡Significantly different from JEV SA14-14-2 by ANOVA with Tukey’s test ($P < 0.05$).
§Significantly different from JEV SA14-14-2 D by ANOVA with Tukey’s test ($P < 0.05$).
had a 10-fold higher IC LD$_{50}$ and mice had a significantly longer, 8 days, median survival time (Table 5 and Figure 3). Similarly, JEV SA14-14-2 variant D differed from variant H by six reversion mutations (Table 1), and had a 17-fold higher IC LD$_{50}$ and mice had an 8-day median survival time (Table 5 and Figure 3). The most attenuated virus was JEV SA14-14-2 variant C, which differed by two unique mutations from variant D (Table 1) that increased the IC LD$_{50}$ level > 60-fold and 80% of mice survived a 10$^2$ PFU dose (Table 5 and Figure 3). Thus, increased amino acid sequence divergence of Vero-adapted JEV SA14-14-2 variants from the reference vaccine strain appears to correlate with increased attenuation in suckling mice.

**DISCUSSION**

The live-attenuated JEV SA14-14-2 vaccine has a proven record of safety and efficacy, but its current production in PHK cells requires maintenance of pathogen-free hamster colonies as a source of kidneys for cell culture, which is a
safety concern and adds to production costs. In addition, several laboratories have sequenced the JEV SA14-14-2 vaccine strain over the last 20 years with significantly different results\textsuperscript{27,28,48} (Table 2), suggesting that the vaccine seed virus is not clonal, and this could affect attenuation and immunogenicity between vaccine lots. It was for these reasons that we initiated the development of a second-generation, recombinant JEV SA14-14-2 live-attenuated vaccine produced in WHO qualified Vero cells.

In this study, we isolated 10 genetically distinct Vero cell-adapted JEV SA14-14-2 variants. Because Vero cells are defective in type I IFN production, unlike PHK cells, a subset of virus variants was isolated after passage in Vero cells treated with a low dose of IFN-α. A single C-S66L reversion mutation was present in all Vero-adapted virus variants and indicated that the dominant strain in the original virus stock containing Ser at amino acid 66, was negatively selected during biological cloning steps in Vero cells. A similar observation was very recently reported by Yang and colleagues\textsuperscript{35} following serial passage of JEV SA14-14-2 in Vero cells. A recombinant JEV mutant (rJEVmut14-14-2) containing a polyprotein amino acid sequence nearly identical to the JEV SA14-14-2 GenBank reference sequence (accession no. JN604986) was constructed and recovered in Vero cells. Although the rJEVmut14-14-2 virus retained the engineered Ser at amino acid 66 after biological cloning steps, six additional passages resulted in reversion of this mutation to Leu, which also significantly increased virus titer. These observations strongly suggest that reversion mutation C-S66L is required for adaptation of JEV SA14-14-2 to replication in Vero cells.

The host type I IFN response is critical for restricting replication and dissemination of flaviviruses\textsuperscript{10,54–57} and JEV SA14-14-2 is capable of inducing morbidity in IFN-deficient mice,\textsuperscript{38} indicating that innate-immune antiviral mechanisms contribute to its attenuated phenotype. Studies in primary human monocyte-derived macrophages showed that JEV SA14-14-2 induced similar levels of type I IFN as WT JEV but is more sensitive to its effects. Therefore, we determined the type I IFN-sensitivity phenotype in Vero cells for the JEV SA14-14-2 derived virus variants generated in this study. Overall, viruses that replicated most effectively in Vero cells, like WT JEV strains, were more resistant to the antiviral effects of type I IFN-treatment. Consequently, reversion mutations like C-S66L and NS2A-V40A that improved replication in Vero cells also appeared to increase resistance to type I IFN-stimulation. The combination of these two reversion mutations in JEV SA14-14-2 variant 3\textsuperscript{IFN} significantly improved type I IFN-resistance in Vero cells to nearly WT JN604986.

### Table 4

<table>
<thead>
<tr>
<th>Virus</th>
<th>IC LD\textsubscript{50} (PFU)</th>
<th>Median survival (days)\textsuperscript{*}</th>
<th>Amino acids different from variant H</th>
<th>IC Number alive/total</th>
<th>IP Number alive/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>rJEVmut14-14-2</td>
<td>10\textsuperscript{4}</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>JEV SA14-14-2</td>
<td>10\textsuperscript{4}</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>JEV SA14-14-2 A</td>
<td>10\textsuperscript{4}</td>
<td>9/9†</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>JEV SA14-14-2 B</td>
<td>10\textsuperscript{4}</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>JEV SA14-14-2 C</td>
<td>10\textsuperscript{4}</td>
<td>9/9†</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>JEV SA14-14-2 D</td>
<td>10\textsuperscript{4}</td>
<td>9/9†</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>JEV SA14-14-2 G</td>
<td>10\textsuperscript{4}</td>
<td>9/9†</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>JEV SA14-14-2 H</td>
<td>10\textsuperscript{4}</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
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<tr>
<td>JEV SA14-14-2 3\textsuperscript{IFN}</td>
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<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Mock</td>
<td>–</td>
<td>0/5</td>
<td>2/5</td>
<td>ND§</td>
<td>ND</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Evaluated for mice inoculated with virus by the IP route.
\textsuperscript{†}A single mouse died of trauma at the injection site.
\textsuperscript{‡}This virus was evaluated in a second, independent experiment.
\textsuperscript{§}ND = not determined.

### Table 5

<table>
<thead>
<tr>
<th>Virus</th>
<th>IC LD\textsubscript{50} (PFU)</th>
<th>Median survival (days)\textsuperscript{*}</th>
<th>Amino acids different from variant H</th>
</tr>
</thead>
<tbody>
<tr>
<td>rJEV India/78</td>
<td>0.2</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td>JEV SA14-14-2 H</td>
<td>0.1</td>
<td>7†</td>
<td>–</td>
</tr>
<tr>
<td>JEV SA14-14-2</td>
<td>0.7</td>
<td>7†</td>
<td>1</td>
</tr>
<tr>
<td>rJEVmut14-14-2</td>
<td>0.8</td>
<td>7†</td>
<td>3</td>
</tr>
<tr>
<td>JEV SA14-14-2 G</td>
<td>1.0</td>
<td>8†‡</td>
<td>4</td>
</tr>
<tr>
<td>JEV SA14-14-2 D</td>
<td>1.7</td>
<td>8†‡</td>
<td>5</td>
</tr>
<tr>
<td>JEV SA14-14-2 C</td>
<td>&gt;100.0</td>
<td>&gt;21§</td>
<td>7</td>
</tr>
<tr>
<td>Mock</td>
<td>NA</td>
<td>&gt;21§</td>
<td>–</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Evaluated for mice inoculated intracerebral (IC) with 10\textsuperscript{6} PFU of virus or mock infected and monitored for 21 days for signs of encephalitis. Survival curves after inoculation with rJEVmut14-14-2, JEV SA14-14-2, and variants C, D, G, and H were statistically different from WT rJEV India/78 (Log-rank test, \( P \leq 0.0001 \)).

\textsuperscript{†}Survival curve is statistically different from rJEV India/78 by Log-rank test.
\textsuperscript{‡}Survival curve is statistically different from JEV SA14-14-2 variant H by Log-rank test.
\textsuperscript{§}Survival curve is statistically different from all other viruses tested by Log-rank test.

\textsuperscript{△}Survival curve is statistically different from all other viruses tested by Log-rank test.
levels. Previously, it was shown that the JEV NS2A protein antagonizes the activity of type I IFN-induced protein kinase R that contributes to inducing an intracellular antiviral state, and mutation NS2A-T33I abolished this activity, decreased virus titer in tissue culture, and reduced virulence in mice.\(^5\) Therefore, mutation NS2A-A40V, which is proximal to NS2A-T33I, might contribute to type I IFN-sensitivity and attenuation of JEV SA14-14-2 by a similar mechanism, but was not critical for attenuation of JEV SA14-14-2 variant \(^3\)IFN in weanling mice.

There were also unique mutations identified in some JEV SA14-14-2 variants that modulated type I IFN-sensitivity in Vero cells and were not linked to improved virus replication. The JEV SA14-14-2 variant \(^2\)IFN had a nearly WT level of resistance to type I IFN-treatment that was produced by mutations E-M61, NS3-V255A, or NS4A-S4G, but lacked the NS2A-V40A mutation associated with IFN resistance in the \(^3\)IFN variant. By contrast, JEV SA14-14-2 variants C and \(^5\)IFN had remarkably high levels of type I IFN-sensitivity produced by mutations E-H394Q or NS3-A160V and NS2B-E27D or NS3-V22A, respectively. Previously, it was shown that a single JEV E protein mutation (E138K) increased virus sensitivity to type I IFN, but this phenotype was coupled with altered levels of virus replication in tissue culture,\(^50\) unlike the E protein mutations in JEV SA14-14-2 variants \(^2\)IFN and C. However, the JEV NS4A protein and the NS2B and NS3 proteins of genetically related West Nile virus Kunjin are known to function as type I IFN-antagonists.\(^61,62\) Therefore, it is likely that one or more unique non-structural protein mutations in JEV SA14-14-2 variants C, \(^2\)IFN and \(^5\)IFN modulated sensitivity to type I IFN in Vero cells.

The virulence phenotype for subsets of Vero-adapted JEV SA14-14-2 variants was tested in weanling and suckling mice. The genetically diverse JEV SA14-14-2 variants were avirulent in weanling mice, even though some had as many as 10 mutations, including seven reversion mutations relative to the reference strain. Of importance, this indicated that mutations acquired during Vero cell-adaptation did not alter the attenuated phenotype of the JEV SA14-14-2 variants in weanling mice, although the variants differed in their level of attenuation in suckling mice. Recombinant JEVmut14-14-2, the JEV SA14-14-2 laboratory stock, and virus variants A, B, C, D, G, H, and \(^3\)IFN were very immunogenic in mice. This was particularly interesting because five of seven reversion mutations (E-A177T, E-H264Q, NS1-H351D, nucleotide change A3599G, and NS5-A671V) in virus variants B, C, D, and \(^3\)IFN were also present in JEV SA14-14-2 PDK,\(^27,30,66\) which was reported to be more immunogenic.\(^27,66\) Genetic differences between these Vero-adapted virus variants and the PDK-adapted virus, including C-S66L, E-K243E, NS3-A223V, NS5-T644N, and NS5-H639Q likely account for their higher level of observed immunogenicity.

There are many advantages to producing viral vaccines in WHO qualified Vero cells, therefore we adapted the JEV SA14-14-2 vaccine strain to replicate in this cell line and isolated a set of Vero-adapted virus variants. The genetic determinants that govern the stable attenuation and high level of immunogenicity of JEV SA14-14-2 are not well understood, which prompted us to assess the \textit{in vitro} and \textit{in vivo} phenotypes of several genetically distinct virus variants. We identified a single reversion mutation (S66L) in the capsid protein that appeared to be essential for Vero cell-adaptation and this mutation significantly improved virus titer in tissue culture without affecting attenuation in weanling mice. The high level of type I IFN-sensitivity exhibited by JEV SA14-14-2 is likely determined by mutations that reduce replicative fitness and also by rare mutations in non-structural proteins that have previously been implicated as type I IFN-antagonists, and might contribute to attenuation. Vero cell-adapted JEV SA14-14-2 variants B, C, D, and \(^3\)IFN had excellent attenuation levels in mice and were highly immunogenic, thereby reducing the possibility of producing vaccine candidates with an inferior phenotype to the current live-attenuated vaccine. Future studies will aim to generate recombinant clones of one or more of these Vero-adapted JEV SA14-14-2 variants with the goal of producing a live-attenuated second-generation vaccine.

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