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NOTE

Biological and Genetic Properties of SA_{14}-14-2, a Live-Attenuated Japanese Encephalitis Vaccine That Is Currently Available for Humans

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Japanese encephalitis virus (JEV), a mosquito-borne flavivirus, is a major cause of acute encephalitis, a disease of significance for global public health. In the absence of antiviral therapy to treat JEV infection, vaccination is the most effective method of preventing the disease. In JE-endemic areas, the most widely used vaccine to date is SA_{14}-14-2, a live-attenuated virus derived from its virulent parent SA_{14}. In this study, we describe the biological properties of SA_{14}-14-2, both *in vitro* and *in vivo*, and report the genetic characteristics of its genomic RNA. In BHK-21 (hamster kidney) cells, SA_{14}-14-2 displayed a slight delay in plaque formation and growth kinetics when compared to a virulent JEV strain, CNU/LP2, with no decrease in maximum virus production. The delay in viral growth was also observed in two other cell lines, SH-SY5Y (human neuroblastoma) and C6/36 (mosquito larva), which are potentially relevant to JEV pathogenesis and transmission. In 3-week-old ICR mice, SA_{14}-14-2 did not cause any symptoms or death after either intracerebral or peripheral inoculation with a maximum dose of up to 1.5×10^3 plaque-forming units (PFU) per mouse. The SA_{14}-14-2 genome consisted of 10977 nucleotides, one nucleotide longer than all the previously reported genomes of SA_{14}-14-2, SA_{14} and two other SA_{14}-derived attenuated viruses. This difference was due to an insertion of one G nucleotide at position 10701 in the 3′ noncoding region. Also, we noted a significant number of nucleotide and/or amino acid substitutions throughout the genome of SA_{14}-14-2, except for the prM protein-coding region, that differed from SA_{14} and/or the other two attenuated viruses. Our results, together with others', provide a foundation not only for the study of JEV virulence but also for the development of new and improved vaccines for JEV.

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Japanese encephalitis virus (JEV) is the causative agent of JE, the most important form of viral encephalitis that affects ~25 countries in Asia (Burke and Leake, 1988; Endy and Nisalak, 2002; Halstead and Jacobsen, 2008). Over the past two decades, JEV has also spread to new geographic locations such as Australia (Hanna *et al.*, 1996, 1999; Mackenzie *et al.*, 2002b), Pakistan (Igarashi *et al.*, 1994), and Saipan (Paul *et al.*, 1993); thus, this virus is now recognized as an emerging pathogen of global public health significance (Mackenzie *et al.*, 2004; Weaver and Barrett, 2004; Mackenzie *et al.*, 2007; Erlanger *et al.*, 2009; van den Hurk *et al.*, 2009; Weaver and Reisen, 2010). JEV is naturally transmitted to humans through the bite of an infected culicine mosquito, most often *Culex tritaeniorhynchus*, and maintained in an enzootic cycle between mosquito vectors and vertebrate amplifying hosts/reservoirs, primarily domestic pigs and wild birds (Rosen, 1986; Burke and Leake, 1988; Endy and Nisalak, 2002; Gubler *et al.*, 2007; Halstead and Jacobsen, 2008). The global incidence of JE is largely unknown, because the current surveillance and detection systems vary significantly throughout the world (Monath, 2002; Solomon and Vaughn, 2002; Solomon and Winter, 2004). Although a minor portion of JEV infections develop into encephalitis, the annual occurrence of JE in Asia is estimated to be in a range of approximately 50,000 to 175,000 cases, depending on age, geographic location, and vaccination status (Burke and Leake, 1988; Tsai, 2000; Campbell *et al.*, 2011). About a quarter of clinical cases are fatal, and up to half of survivors live with permanent neuropsychiatric complications (Tsai, 2000; Solomon, 2006; WHO, 2006).

JEV is a member of the genus *Flavivirus* in the family *Flaviviridae*. Within the genus, JEV represents the JEV serological group that also includes several neurotropic flaviviruses, e.g., West Nile (WN), Murray Valley encephalitis, and St. Louis encephalitis viruses, and shows a close genetic relationship with other human pathogens, such as dengue (DEN), yellow fever (YF), and tick-borne encephalitis viruses (Mackenzie *et al.*, 2002a; Calisher and Gould, 2003; Thiel *et al.*, 2005). JEV is an enveloped virus with an ~11-kb, linear plus-strand genomic RNA that contains a 5′ cap structure but lacks a 3′ poly(A) tail (Rice *et al.*, 1985; Sumiyoshi *et al.*, 1987; Yun *et al.*, 2003a). The genome encodes a single long open reading frame (ORF) flanked by short noncoding regions at its 5′ and 3′ ends (Lindenbach and Rice, 2003;

Yun and Lee, 2006; Lindenbach *et al*., 2007). Our understanding of JEV genome replication and expression is largely based on previous work with other flaviviruses, i.e., YFV, WNV, and DENV. Upon viral entry into susceptible cells, the genomic RNA is translated to yield a polyprotein, which is processed by viral and cellular proteases into at least 10 functional proteins: three structural (C, prM, and E) and seven nonstructural proteins (NS1, 2A, 2B, 3, 4A, 4B, and 5) (Chambers *et al*., 1990; Yun and Lee, 2006). The structural proteins constitute an infectious virion (Kuhn *et al*., 2002; Mukhopadhyay *et al*., 2003; Zhang *et al*., 2003) and participate in viral entry and assembly (Mukhopadhyay *et al*., 2005; Harrison, 2008). The nonstructural proteins function in viral RNA replication (Brinton, 2002; Westaway *et al*., 2002; Markoff, 2003; Villordo and Gamarnik, 2009; Paranjape and Harris, 2010), viral assembly (Kümmerer and Rice, 2002; Liu *et al*., 2003; Pijlman *et al*., 2006; Leung *et al*., 2008; Patkar and Kuhn, 2008), and/or evasion of host innate immunity (Muñoz-Jordan *et al*., 2003, 2005; Guo *et al*., 2005; Liu *et al*., 2005; Diamond, 2009; Robertson *et al*., 2009). For RNA replication, the two largest nonstructural proteins possess multiple enzymatic activities: NS3 acts as a serine protease (together with its cofactor NS2B) (Chambers *et al*., 1991; Falgout *et al*., 1993), an RNA-stimulated nucleoside triphosphatase (Wengler and Wengler, 1991), an RNA helicase (Li *et al*., 1999), and an RNA triphosphatase (Wengler and Wengler, 1993). NS5 functions as a methyltransferase (Egloff *et al*., 2002; Ray *et al*., 2006), an RNA guanylyltransferase (Issur *et al*., 2009), and an RNA-dependent RNA polymerase (Tan *et al*., 1996; Ackermann and Padmanabhan, 2001).

Four types of JE vaccines are in local use in different regions of the world (Monath, 2002; Beasley *et al*., 2008; Jelinek, 2008; Halstead and Thomas, 2010; Wilder-Smith and Halstead, 2010; Halstead and Thomas, 2011): (*i*) the inactivated mouse brain-derived vaccine based on the Nakayama or Beijing-1 strain, (*ii*) the inactivated cell culture-derived vaccine based on the Beijing-3 or SA_{14}-14-2 strain, (*iii*) the live-attenuated cell culture-derived vaccine based on the SA_{14}-14-2 strain, and (*iv*) the live chimeric vaccine (Lai and Monath, 2003) based on a recombinant YFV 17D in which the prM and E protein-coding region of YFV 17D has been replaced with the corresponding region of JEV SA_{14}-14-2 (Chambers *et al*., 1999; Guirakhoo *et al*., 1999; Monath *et al*., 1999; Monath *et al*., 2000; Arroyo *et al*., 2001). Of these, only two are currently licensed internationally: the formalin-inactivated Nakayama and live-attenuated SA_{14}-14-2 vaccines. The inactivated Nakayama vaccine was produced in many Asian countries, but its production has now been scaled down or discontinued (Fischer *et al*., 2010) because of the availability of new and improved JE vaccines (Sakaguchi *et al*., 2001; Plesner, 2003; Solomon, 2006). To date, the live SA_{14}-14-2 vaccine is the only internationally licensed vaccine available in sufficient quantity, constituting more than 50% of the total global production (WHO, 2005). The SA_{14}-14-2 vaccine virus was developed empirically by serial passage of its virulent parental virus, SA_{14}, in cell cultures (e.g., primary hamster kidney cells) and in animals (e.g., mice and hamsters) (Yu, 2010). Since its initial licensure in China in 1989, this vaccine has been administered to >300 million children in China and several Asian countries as well (i.e., Nepal, South Korea, India, Sri Lanka, and Thailand), with no report of vaccine-related adverse JE cases (Xin *et al*., 1988; Liu *et al*., 1997; Sohn *et al*., 1999). The immunogenicity, safety, and long-term protective efficacy of the SA_{14}-14-2

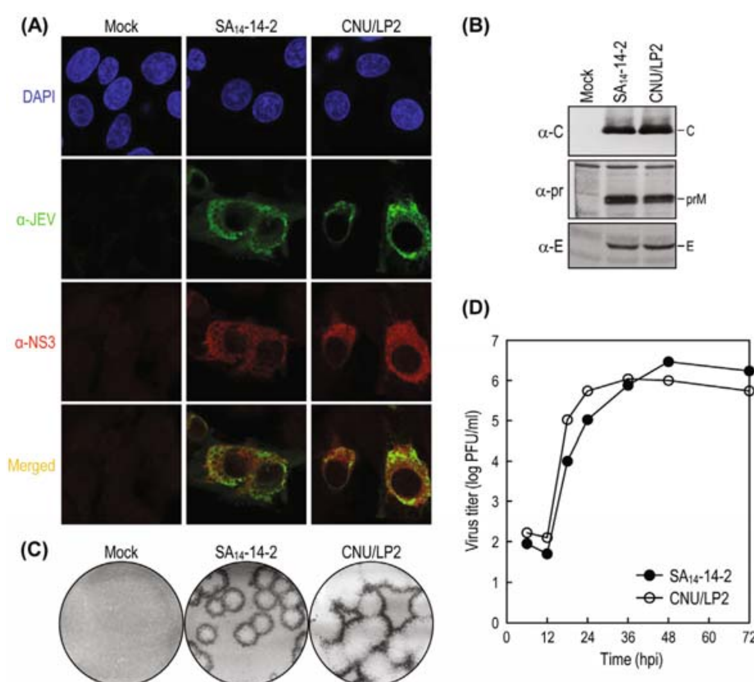

Fig. 1. Replication of JEV SA_{14}-14-2 and CNU/LP2 strains in BHK-21 cells. (A) Detection of JEV proteins. Cells were mock-infected or infected with SA_{14}-14-2 or CNU/LP2; 20 h later, they were fixed and stained with a primary antibody, either JEV-specific mouse hyperimmune ascites (α-JEV) or a polyclonal rabbit anti-NS3 antiserum (α-NS3), and subsequently with a secondary antibody, either an FITC-conjugated goat anti-mouse IgG (green fluorescence) or Cy3-conjugated goat anti-rabbit IgG (red fluorescence). Cell nuclei were visualized by staining with DAPI (blue fluorescence). Images were obtained with a LSM-710 confocal microscope. Merged images are also provided. (B) Expression of JEV structural proteins. Cells were mock-infected or infected at an m.o.i of 1 PFU/cell with SA_{14}-14-2 or CNU/LP2 for 20 h. In each case, an equal portion of total cell lysate was separated on 15% Tricine-SDS-PAGE (for the detection of both C and prM proteins) or 10% glycine-SDS-PAGE (for the detection of E protein). Viral proteins were visualized by immunoblotting with a panel of three polyclonal rabbit antisera, each specific for JEV C, prM, and E proteins, followed by an AP-conjugated goat anti-rabbit IgG. Viral proteins were stained by incubating with a mixture of BCIP and NBT as a substrate. (C) Morphology of JEV foci/plaques. Cells were infected with SA_{14}-14-2 or CNU/LP2 and overlaid with agarose for 4 days. Following fixation, cells were immunostained with JEV-specific mouse hyperimmune ascites and a peroxidase-conjugated goat anti-mouse IgG, and stained with diaminobenzidine substrate. (D) Kinetics of JEV growth. Cells were infected at an m.o.i of 1 with SA_{14}-14-2 or CNU/LP2. Cell culture supernatants were collected at the indicated time points, and virus titers were determined by plaque assays on BHK-21 cells. The growth curve represents one of two independent experiments yielding similar results.

vaccine are reported to be promising for its extended use in global immunization (Hennessy *et al.*, 1996; Tsai *et al.*, 1998; Sohn *et al.*, 1999; Bista *et al.*, 2001; Ohrr *et al.*, 2005; Tandan *et al.*, 2007; Sohn *et al.*, 2008; Liu *et al.*, 2011). In South Korea, the SA_{14}-14-2 vaccine was introduced in 2002, with a recommended immunization schedule of two doses administered 12 months apart in children aged 1–2 years and a booster at 6 years of age (WHO, 2008).

In this work, we aimed to characterize the biological properties of SA_{14}-14-2, both *in vitro* and *in vivo*, and to define the genetic characteristics of its genomic RNA. A stock of SA_{14}-14-2 virus was retrieved directly from a batch of commercial vaccine vials and used throughout the entire course of this study, in order to avoid any potential for its adaptation that could occur during propagation in cell cultures. Initially, we analyzed the virological properties of SA_{14}-14-2 *in vitro*, in parallel with a highly virulent JEV strain CNU/LP2 (Yun *et al.*, 2003b; Kang *et al.*, 2004; Kim *et al.*, 2008), in cell culture systems. In BHK-21 (baby Syrian hamster kidney) cells, confocal microscopy with JEV-specific mouse hyperimmune ascites revealed that at 20 h post-infection (hpi), the viral proteins expressed in SA_{14}-14-2-infected cells were predominantly localized around the perinuclear membranes, and their localization was indistinguishable from that of the virus in CNU/LP2-infected cells (Fig. 1A, α-JEV). Identical results were obtained with a polyclonal rabbit antiserum specific for the JEV NS3 protein (Fig. 1A, α-NS3). Also, immunoblot analyses with a panel of three polyclonal rabbit antisera, each recognizing JEV C (α-C), prM (α-pr), and E (α-E) proteins, showed that the three viral structural proteins accumulated in SA_{14}-14-2-infected cells at 20 hpi were all comparable in size and amount to those produced in CNU/LP2-infected cells (Fig. 1B). On the other hand, direct staining of cell monolayers with the JEV-specific mouse hyperimmune ascites indicated that at 4 days post-infection (dpi), the size of foci/plaques formed by SA_{14}-14-2 was on average ~35% smaller than that of foci/plaques produced by CNU/LP2 (Fig. 1C). Consistent with this finding, the viral growth of SA_{14}-14-2 was slightly slower than that of CNU/LP2, with no decrease in maximum virus production, when cells were infected at a multiplicity of infection (m.o.i) of 1 with each virus (Fig. 1D). The delay in viral growth was also observed in two other cell lines, SH-SY5Y (human neuroblastoma) and C6/36 (*Aedes albopictus* mosquito larvae), which are potentially relevant to JEV pathogenesis and transmission, respectively (data not shown).

We next examined the virulence of SA_{14}-14-2 *in vivo*, by estimating the 50% lethal dose (LD_{50}) in a murine infection model system. The LD_{50} values of the SA_{14}-14-2 and CNU/LP2 (reference) viruses were determined by inoculating groups of 3-week-old female ICR mice (*n*=10 per group) by either the intracerebral (i.c) or the intramuscular (i.m) route as described previously (Kim *et al.*, 2008). Each virus stock was serially diluted 10-fold in minimal essential medium (MEM), and 20 μl (for i.c) or 50 μl (for i.m) of each dilution was used to inoculate each mouse. For each route, a control group (*n*=10) was inoculated with an equivalent volume of MEM. Mice were observed every 12 h for 24 days for the development of any JEV-induced clinical signs (ruffled fur, hunched posture, tremors, and hind limb paralysis) and death. In agreement with our previous findings (Kim *et al.*, 2008), CNU/LP2 was highly neurovirulent and neuroinvasive, yielding an LD_{50} of <1.5 PFU for both the i.c and i.m routes of inoculation (Table 1, CNU/LP2). All dead mice developed the symptoms of JEV infection, and viral replication in their brain tissues was confirmed by virus titration after the experiment (data not shown). In contrast, SA_{14}-14-2 was highly attenuated, as shown by the observation that all the infected mice remained healthy and showed no clinical signs of viral infection after either the i.c or i.m route of inoculation with a maximum dose of up to 1.5×10^3 PFU/mouse (Table 1, SA_{14}-14-2). In the case of five mice inoculated by the i.c route with a dose of 1.5×10^3 PFU, we performed immunohistochemical staining with a polyclonal rabbit antiserum specific for the JEV NS1 protein and found that only a small number, if any, of the NS1-immunoreactive neurons were localized in any region of the brain infected with SA_{14}-14-2 (Yun SI and Lee YM, manuscript in preparation). As expected, the control groups of mock-infected mice all survived with no signs of disease (data not shown).

In an effort to characterize the genetic properties of SA_{14}-14-2, we determined the complete nucleotide sequence of its genomic RNA. Template RNA was extracted directly from a batch of the commercial vaccine vials distributed in South Korea in 2005. Each of the vials was estimated to contain ~1×10^5 PFU by plaque titration on BHK-21 cells. The extracted RNA was subjected to cDNA synthesis and PCR amplification according to our previously established protocols (Yun *et al.*, 2003a, 2003b). In sum, a total of three overlapping cDNA fragments that covered the entire viral genome except the utmost 5′ and 3′ termini were amplified and directly sequenced on both strands. The 3′-terminal sequences were identified by a 3′ RACE protocol, ligating a

Table 1. **Pathogenicity of JEV SA_{14}-14-2 and CNU/LP2 strains in 3-week-old ICR mice**

Virus	Inoculum (PFU/mouse)	i.c				i.m			
		Alive	Dead	Total	LD_{50} (PFU)	Alive	Dead	Total	LD_{50} (PFU)
CNU/LP2	1.5×10^3	0	10	10	<1.5	0	10	10	<1.5
	1.5×10^2	0	10	10		0	10	10	
	1.5×10^1	0	10	10		2	8	10	
	1.5	2	8	10		3	7	10	
SA_{14}-14-2	1.5×10^3	10	0	10	>1.5×10^3	10	0	10	>1.5×10^3
	1.5×10^2	10	0	10		10	0	10	
	1.5×10^1	10	0	10		10	0	10	
	1.5	10	0	10		10	0	10	

Table 2. Genome organization of JEV SA$_{14}$-14-2 strain

Locus/Protein	Nucleotide[a]			Amino acid[b]		
	Start	End	Length (nt)[c]	Start	End	Length (aa)[c]
5′ NCR	1	95	95			
C	96	476	381	1	127	127
prM	477	977	501	128	294	167
E	978	2477	1500	295	794	500
NS1	2478	3533	1056	795	1146	352
NS2A	3534	4214	681	1147	1373	227
NS2B	4215	4607	393	1374	1504	131
NS3	4608	6464	1857	1505	2123	619
NS4A	6465	6911	447	2124	2272	149
NS4B	6912	7676	765	2273	2527	255
NS5	7677	10394	2718	2528	3433	906
3′ NCR	10395	10977	583			
Total			10977			3433

[a] Nucleotide positions refer to the complete genome sequence of JEV SA$_{14}$-14-2 (GenBank accession no. JN604986).
[b] Amino acid positions refer to the precursor polyprotein sequence of JEV SA$_{14}$-14-2 (GenBank accession no. JN604986).
[c] nt, nucleotide; aa, amino acid.

synthetic oligonucleotide to the 3′-end of the genomic RNA to provide a specific primer-binding site during RT-PCR. The 5′-terminal sequences were identified by self-ligation of the genomic RNA and RT-PCR amplification of the ligated 3′-5′ region. In both termini, the consensus sequences were determined by cloning of the cDNA amplicons and sequencing of >40 randomly picked independent clones. The full genome consensus sequence of SA$_{14}$-14-2 was deposited in GenBank with the accession number JN604986. As summarized in Table 2, the SA$_{14}$-14-2 genomic RNA is 10977 nucleotides in length and consists of a 95-nucleotide 5′NCR, a 10299-nucleotide ORF, and a 583-nucleotide 3′NCR. The ORF has a coding capacity of 3433 amino acids (including the stop codon). The genetic loci of the three structural and seven nonstructural proteins were predicted by comparing the deduced amino acid sequences with the known cleavage sites of other flaviviruses (Chambers *et al.*, 1990).

Finally, we compared the complete nucleotide and deduced amino acid sequences of the genomes between the virulent parent SA$_{14}$ and three SA$_{14}$-derived attenuated viruses (i.e., SA$_{14}$-2-8, SA$_{14}$-12-1-7, and SA$_{14}$-14-2). Of particular concern was the fact that the genomes of both SA$_{14}$ and SA$_{14}$-14-2 have been sequenced by several independent groups, and their nucleotide sequences are not identical, mainly because of variations in the cultivation history of the viruses. In our comparative sequence analyses, we therefore included a total of eight genomic sequences currently retrievable from GenBank: (*i*) three for SA$_{14}$, designated SA$_{14}$ Seq1 (M55506; Nitayaphan *et al.*, 1990), SA$_{14}$ Seq2 (D90194; Aihara *et al.*, 1991), and SA$_{14}$ Seq3 (U14163; Ni *et al.*, 1994, 1995); (*ii*) one for SA$_{14}$-2-8 (U15763; Ni *et al.*, 1995); (*iii*) one for SA$_{14}$-12-1-7 (AF416457); and (*iv*) three for SA$_{14}$-14-2, designated SA$_{14}$-14-2 Seq1 (AF315119), SA$_{14}$-14-2 Seq2 (D90195; Aihara *et al.*, 1991), and SA$_{14}$-14-2 Seq3 (JN604986, this study). Nucleotide and amino acid sequence alignments were carried out using Clustal W. The nucleotide and amino acid sequence differences between the genomes of SA$_{14}$ and three SA$_{14}$-derived attenuated viruses are summarized in Table 3. One unexpected finding is that the 10977-nucleotide genome of SA$_{14}$-14-2 determined in the present study was one nucleotide longer than any previously reported genomes of SA$_{14}$, SA$_{14}$-2-8, SA$_{14}$-12-1-7, and SA$_{14}$-14-2 (which are all 10976 nucleotides). This difference was due to an insertion of one G nucleotide at position 10701 in the 3′NCR. In addition, we also noted a total of 123 nucleotides (59 amino acids, solid squares) that vary in one or more of the eight genomic sequences we used for analysis; they were distributed throughout the entire genome of SA$_{14}$-14-2, except for the prM protein-coding region. Of these, a panel of 39 nucleotides (16 amino acids, open squares) was invariably different between the genomes of SA$_{14}$ and SA$_{14}$-14-2, regardless of which version of the genomic sequences was used for comparison: 1 in the 5′NCR (^{39}A); 1 in C (^{292}C [^{66}S]); 7 in E (^{1061}C, ^{1296}U [^{401}F], ^{1389}A [^{432}K], ^{1503}G [^{470}V], ^{1506}G [^{471}A], ^{1769}U [^{558}H], and ^{1813}U [^{573}M]); 1 in NS1 (^{3528}C [^{1145}H]); 3 in NS2A (^{3776}U, ^{3801}U, and ^{4106}G); 2 in NS2B (^{4403}U [^{1436}D] and ^{4408}G [^{1438}G]); 6 in NS3 (^{4782}G [^{1563}V], ^{4825}A [^{1577}K], ^{4921}G [^{1609}G], ^{4922}C [^{1609}G], ^{6008}U, and ^{6425}G); 1 in NS4A (^{6728}A); 4 in NS4B (^{6944}G, ^{7121}U, ^{7193}U, and ^{7227}G [^{2378}V]); 12 in NS5 (^{7736}U, ^{8099}U, ^{8394}U, ^{8832}U [^{2913}Y], ^{8882}U, ^{8891}U, ^{9688}C [^{3198}A], ^{9695}A, ^{9818}U, ^{10046}A, ^{10139}U, and ^{10217}C); and 1 in the 3′NCR (^{10428}C). Given their consistency, a subset of these nucleotide and/or amino acid changes is presumably responsible for the attenuation phenotype of SA$_{14}$-14-2; however, other genetic changes found in one or more of the eight genomic sequences might also be associated with the attenuation of SA$_{14}$-14-2.

In this work, we report the biological and genetic properties of SA$_{14}$-14-2, a live-attenuated JE vaccine that is currently available for humans in China and five other Asian countries. We found that SA$_{14}$-14-2 replicated with a high efficiency but exhibited a delay in viral growth in three different cell lines (i.e., BHK-21, SH-SY5Y, and C6/36), relative to the highly virulent JEV strain CNU/LP2. The delayed viral growth was more evident in the appearance of foci/plaques. In BHK-21 cells, the focus/plaque sizes for SA$_{14}$-14-2 were significantly smaller than those produced by CNU/LP2. These results are in agreement with previous reports (Eckels

Table 3. Differences in complete nucleotide and deduced amino acid sequences of the genomic RNAs among the JEV SA_{14}, SA_{14}-2-8, SA_{14}-12-1-7, and SA_{14}-14-2 strains

Region	Position		SA_{14}			SA_{14}-2-8	SA_{14}-12-1-7	SA_{14}-14-2			
	NT	AA	Seq1[a]	Seq2[a]	Seq3[a]	Seq[b]	Seq[b]	Seq1[c]	Seq2[c]	Seq3[c]	
5'NCR	21		U	U	U	U	C	U	U	U	
5'NCR	**39**		**U**	**U**	**U**	**U**	**A**	**A**	**A**	**A**	
C	127	11	A(N)[d]	A(N)	A(N)	A(N)	G(S)	A(N)	A(N)	A(N)	■
C	**292**	**66**	**U(L)**	**U(L)**	**U(L)**	**U(L)**	**C(S)**	**C(S)**	**C(S)**	**C(S)**	■□
C	316	74	A(K)	A(K)	A(K)	A(K)	G(R)	A(K)	A(K)	A(K)	■
C	375	94	G(A)	G(A)	G(A)	G(A)	A(T)	G(A)	G(A)	G(A)	■
E	1017	308	G(G)	G(G)	G(G)	G(G)	A(R)	G(G)	G(G)	G(G)	■
E	1052	319	A(L)	A	G	A	A	A	A	A	
E	**1061**	**322**	**U(D)**	**U**	**U**	**C**	**C**	**C**	**C**	**C**	
E	1217	374	C(A)	U	C	C	C	C	C	C	
E	**1296**	**401**	**C(L)**	**C(L)**	**C(L)**	**C(L)**	**U(F)**	**U(F)**	**U(F)**	**U(F)**	■□
E	1354	420	U(I)	U(I)	U(I)	C(T)	U(I)	U(I)	U(I)	U(I)	■
E	1360	422	G(R)	G(R)	G(R)	A(K)	G(R)	G(R)	G(R)	G(R)	■
E	**1389**	**432**	**G(E)**	**G(E)**	**G(E)**	**A(K)**	**A(K)**	**A(K)**	**A(K)**	**A(K)**	■□
E	**1503**	**470**	**A(I)**	**A(I)**	**A(I)**	**G(V)**	**G(V)**	**G(V)**	**G(V)**	**G(V)**	■□
E	**1506**	**471**	**A(T)**	**A(T)**	**A(T)**	**A(T)**	**A(T)**	**G(A)**	**G(A)**	**G(A)**	■□
E	1512	473	A(K)	A(K)	A(K)	G(E)	A(K)	A(K)	A(K)	A(K)	■
E	1532	479	A(E)	A	A	A	G	A	A	A	
E	1661	522	U(P)	U	U	C	U	U	U	U	
E	1708	538	G(G)	G(G)	A(E)	G(G)	G(G)	G(G)	G(G)	G(G)	■
E	**1769**	**558**	**G(Q)**	**G(Q)**	**G(Q)**	**G(Q)**	**U(H)**	**U(H)**	**U(H)**	**U(H)**	■□
E	**1813**	**573**	**A(K)**	**A(K)**	**A(K)**	**A(K)**	**A(K)**	**U(M)**	**U(M)**	**U(M)**	■□
E	1921	609	U(V)	C(A)	C(A)	U(V)	C(A)	U(V)	U(V)	U(V)	■
E	1977	628	C(P)	U(S)	C(P)	C(P)	C(P)	C(P)	C(P)	C(P)	■
E	2012	639	C(L)	C	C	C	U	C	C	C	
E	2051	652	C(N)	C	C	U	C	C	C	C	
E	2293	733	G(R)	A(K)	A(K)	G(R)	G(R)	G(R)	G(R)	G(R)	■
E	2317	741	G(G)	G(G)	G(G)	G(G)	G(G)	A(D)	G(G)	G(G)	■
E	2441	782	A(G)	G	G	A	G	A	A	A	
NS1	2691	866	A(R)	C	A	A	A	A	A	A	
NS1	2843	916	C(I)	U	C	U	C	C	C	C	
NS1	3085	997	A(E)	A(E)	A(E)	G(G)	A(E)	A(E)	A(E)	A(E)	■
NS1	3184	1030	U(V)	U(V)	U(V)	C(A)	U(V)	U(V)	U(V)	U(V)	■
NS1	3284	1063	U(D)	U	U	C	U	U	U	U	
NS1	3290	1065	U(N)	U	U	C	U	U	U	U	
NS1	3351	1086	A(S)	G(G)	A(S)	G(G)	G(G)	A(S)	A(S)	A(S)	■
NS1	3493	1133	U(M)	G(R)	G(R)	G(R)	G(R)	U(M)	U(M)	U(M)	■
NS1	3516	1141	A(R)	C	A	A	A	A	A	A	
NS1	**3528**	***1145**	**G(D)**	**G(D)**	**G(D)**	**G(D)**	**G(D)**	**C(H)**	**C(H)**	**C(H)**	■□
NS1	3530	*1145	U(D)	U(D)	U(D)	U(D)	C(D)	U(H)	U(H)	U(H)	■
NS2A	3535	1147	U(F)	U(F)	U(F)	C(S)	U(F)	U(F)	U(F)	U(F)	■
NS2A	3539	1148	A(K)	U(N)	U(N)	U(N)	A(K)	A(K)	A(K)	A(K)	■
NS2A	3584	1163	U(F)	U	U	C	U	U	U	U	
NS2A	3599	1168	A(E)	G	G	A	A	A	A	A	
NS2A	3652	1186	U(V)	C(A)	C(A)	C(A)	C(A)	U(V)	U(V)	U(V)	■
NS2A	3677	1194	U(G)	C	C	C	U	U	U	U	
NS2A	**3776**	**1227**	**C(A)**	**C**	**C**	**U**	**U**	**U**	**U**	**U**	
NS2A	**3801**	**1236**	**C(L)**	**C**	**C**	**U**	**U**	**U**	**U**	**U**	
NS2A	3849	1252	A(I)	G(V)	G(V)	G(V)	G(V)	G(V)	G(V)	G(V)	■
NS2A	3929	1278	C(A)	C	C	C	C	C	U	C	
NS2A	**4106**	**1337**	**A(A)**	**A**	**A**	**A**	**A**	**G**	**G**	**G**	
NS2B	4250	1385	A(G)	A	A	A	G	A	A	A	
NS2B	4402	*1436	A(E)	A(E)	A(E)	G(G)	A(E)	A(D)	A(D)	A(D)	■
NS2B	**4403**	***1436**	**G(E)**	**G(E)**	**G(E)**	**G(G)**	**G(E)**	**U(D)**	**U(D)**	**U(D)**	■□
NS2B	**4408**	**1438**	**A(D)**	**A(D)**	**A(D)**	**A(D)**	**G(G)**	**G(G)**	**G(G)**	**G(G)**	■□
NS2B	4475	1460	G(L)	G(L)	G(L)	G(L)	G(L)	C(F)	G(L)	G(L)	■
NS2B	4519	1475	U(M)	U(M)	C(T)	U(M)	U(M)	U(M)	U(M)	U(M)	■
NS3	**4782**	**1563**	**A(M)**	**A(M)**	**A(M)**	**A(M)**	**G(V)**	**G(V)**	**G(V)**	**G(V)**	■□
NS3	**4825**	**1577**	**G(R)**	**G(R)**	**G(R)**	**G(R)**	**A(K)**	**A(K)**	**A(K)**	**A(K)**	■□
NS3	**4921**	***1609**	**C(A)**	**C(A)**	**C(A)**	**U(V)**	**G(G)**	**G(G)**	**G(G)**	**G(G)**	■□
NS3	**4922**	***1609**	**U(A)**	**U(A)**	**U(A)**	**C(V)**	**C(G)**	**C(G)**	**C(G)**	**C(G)**	■□
NS3	5230	1712	U(I)	U(I)	U(I)	U(I)	C(T)	U(I)	U(I)	U(I)	■
NS3	5234	1713	U(I)[d]	C	U	U	U	U	U	U	
NS3	5243	1716	U(A)	U	C	U	U	U	U	U	
NS3	5311	1739	C(A)	U(V)	C(A)	C(A)	C(A)	U(V)	U(V)	C(A)	■
NS3	5634	1847	A(R)	A(R)	A(R)	A(R)	A(R)	A(R)	U(W)	A(R)	■
NS3	5994	1967	G(G)	G(G)	G(G)	G(G)	A(S)	G(G)	G(G)	G(G)	■
NS3	**6008**	**1971**	**C(N)**	**C**	**C**	**C**	**U**	**U**	**U**	**U**	
NS3	**6425**	**2110**	**A(Q)**	**A**	**A**	**A**	**G**	**G**	**G**	**G**	
NS4A	6634	2180	U(I)	U(I)	U(I)	U(I)	U(I)	U(I)	C(T)	U(I)	■
NS4A	6700	*2202	A(K)	A(K)	G(R)	G(R)	A(K)	A(K)	A(K)	A(K)	■
NS4A	6701	*2202	G(K)	G(K)	G(R)	A(R)	G(K)	G(K)	G(K)	G(K)	■
NS4A	**6728**	**2211**	**G(T)**	**G**	**G**	**G**	**G**	**A**	**A**	**A**	
NS4A	6904	2270	U(V)	U(V)	U(V)	U(V)	C(A)	U(V)	U(V)	U(V)	■
NS4B	**6944**	**2283**	**A(A)**	**A**	**A**	**A**	**A**	**G**	**G**	**G**	
NS4B	7005	2304	A(M)	A(M)	A(M)	A(M)	G(V)	A(M)	A(M)	A(M)	■
NS4B	**7121**	**2342**	**C(A)**	**C**	**C**	**C**	**U**	**U**	**U**	**U**	
NS4B	**7193**	**2366**	**C(T)**	**C**	**C**	**C**	**U**	**U**	**U**	**U**	
NS4B	**7227**	**2378**	**A(I)**	**A(I)**	**A(I)**	**G(V)**	**G(V)**	**G(V)**	**G(V)**	**G(V)**	■□
NS4B	7337	2414	G(A)	G	G	A	G	G	G	G	
NS4B	7655	2520	U(A)	U	U	U	U	U	G	U	
NS5	7706	2537	G(E)	G(E)	G(E)	U(D)	G(E)	G(E)	G(E)	G(E)	■
NS5	**7736**	**2547**	**C(S)**	**C**	**C**	**C**	**C**	**U**	**U**	**U**	
NS5	7751	2552	U(F)	U	U	C	U	U	U	U	
NS5	7768	2558	C(A)	C(A)	C(A)	C(A)	C(A)	G(G)	C(A)	C(A)	■
NS5	7805	2570	U(A)	C	C	C	C	C	C	C	
NS5	7809	2572	C(R)	C(R)	C(R)	C(R)	C(R)	A(S)	C(R)	C(R)	■
NS5	7871	2592	U(L)	C	C	C	C	U	C	C	
NS5	7926	2611	C(R)	C(R)	C(R)	C(R)	U(C)	C(R)	C(R)	C(R)	■
NS5	8067	2658	G(D)	G(D)	G(D)	G(D)	C(H)	G(D)	G(D)	G(D)	■
NS5	**8099**	**2668**	**C(D)**	**C**	**C**	**C**	**U**	**U**	**U**	**U**	
NS5	8261	2722	G(M)	G(M)	G(M)	G(M)	G(M)	U(I)	G(M)	G(M)	■
NS5	8276	2727	C(R)	C	C	U	C	C	U	U	
NS5	**8394**	**2767**	**C(L)**	**C**	**C**	**U**	**U**	**U**	**U**	**U**	
NS5	8658	2855	G(E)	A(K)	A(K)	A(K)	A(K)	A(K)	A(K)	A(K)	■
NS5	**8832**	**2913**	**C(H)**	**C(H)**	**C(H)**	**C(H)**	**C(H)**	**U(Y)**	**U(Y)**	**U(Y)**	■□
NS5	**8882**	**2929**	**A(I)**	**A**	**A**	**U**	**A**	**U**	**U**	**U**	
NS5	**8891**	**2932**	**C(V)**	**C**	**C**	**U**	**U**	**U**	**U**	**U**	
NS5	9593	3166	G(Q)	G(Q)	G(Q)	G(Q)	G(Q)	U(H)	G(Q)	U(H)	■
NS5	9602	3169	A(R)	G	G	G	G	G	G	G	
NS5	9603	3170	A(K)	A(K)	A(K)	G(E)	A(K)	A(K)	A(K)	A(K)	■
NS5	9607	3171	C(T)	A(N)	A(N)	A(N)	A(N)	A(N)	A(N)	A(N)	■
NS5	**9688**	**3198**	**U(V)**	**U(V)**	**U(V)**	**U(V)**	**U(V)**	**C(A)**	**C(A)**	**C(A)**	■□
NS5	**9695**	**3200**	**G(K)**	**G**	**G**	**G**	**A**	**A**	**A**	**A**	
NS5	**9818**	**3241**	**C(C)**	**C**	**C**	**C**	**U**	**U**	**U**	**U**	
NS5	9824	3243	C(N)	C	C	U	C	C	C	C	
NS5	9898	3268	G(G)	A(D)	G(G)	G(G)	G(G)	G(G)	G(G)	G(G)	■
NS5	9917	3274	A(P)	A	A	A	A	A	A	U	
NS5	9954	3287	G(A)	G(A)	G(A)	G(A)	G(A)	C(P)	G(A)	G(A)	■
NS5	9971	3292	G(Q)	G	G	G	G	A	G	G	
NS5	9978	3295	C(L)	C(L)	C(L)	C(L)	C(L)	G(V)	C(L)	C(L)	■
NS5	9995	3300	U(H)	U	U	U	U	C	U	U	
NS5	**10046**	**3317**	**G(V)**	**G**	**G**	**G**	**A**	**A**	**A**	**A**	
NS5	**10139**	**3348**	**C(V)**	**C**	**C**	**C**	**U**	**U**	**U**	**U**	
NS5	**10217**	**3374**	**U(R)**	**U**	**U**	**U**	**C**	**C**	**C**	**C**	
3'NCR	**10428**		**U**	**U**	**U**	**U**	**U**	**C**	**C**	**C**	
3'NCR	10551		G	G	G	G	A	G	G	G	
3'NCR	10574		C	C	C	C	C	U	C	C	
3'NCR	10701		-	-	-	-	-	-	-	G	
3'NCR	10702		C	C	C	C	G	C	C	C	
3'NCR	10785		C	C	C	C	C	C	U	C	
3'NCR	10950		G	G	G	G	C	C	G	G	
3'NCR	10951		C	C	C	C	G	G	C	C	
3'NCR	10956		G	G	A	G	G	G	G	G	

[a] SA_{14} Seq1 (M55506; Nitayaphan *et al.*, 1990), SA_{14} Seq2 (D90194; Aihara *et al.*, 1991), SA_{14} Seq3 (U14163; Ni *et al.*, 1994, 1995).
[b] SA_{14}-2-8 Seq (U15763; Ni *et al.*, 1995), SA_{14}-12-1-7 Seq (AF416457).
[c] SA_{14}-14-2 Seq1 (AF315119), SA_{14}-14-2 Seq2 (D90195; Aihara *et al.*, 1991), SA_{14}-14-2 Seq3 (JN604986, this study).
[d] Amino acid changes are indicated by the standard single-letter code in parentheses.

et al., 1988; Aihara *et al*., 1991; Yu, 2010). In mice, we confirmed that SA_{14}-14-2 was highly attenuated, both in neurovirulence and in neuroinvasiveness, in accordance with earlier studies using mice and rhesus monkeys (Eckels *et al*., 1988; Aihara *et al*., 1991; Hase *et al*., 1993). On the other hand, in an independent pilot experiment with a high dose of SA_{14}-14-2 (i.e., 1.5×10^4 and 1.5×10^5 PFU/mouse), we found that ~10–20% of the infected mice developed the typical signs of JEV infection and death when inoculated via the i.c route, but not via the i.m route (Song BH, Yun SI, and Lee YM, unpublished data). One important issue with SA_{14}-14-2 remains a risk for reversion of the virus to high virulence. In recent years, SA_{14}-14-2 has been used to produce a new Vero cell-derived inactivated vaccine that has been approved in the US, Europe, Canada, and Australia (Jelinek, 2009; Kollaritsch *et al*., 2009; Fischer *et al*., 2010; CDC, 2011). Also, the prM and E genes of SA_{14}-14-2 have been used to replace the corresponding genes of YFV 17D (Chambers *et al*., 1999), engineering a live-attenuated chimeric YF/JE vaccine (Guirakhoo *et al*., 1999; Monath *et al*., 1999, 2000) that has been licensed in Australia since 2010 (Halstead and Thomas, 2011). Despite the increasing application of SA_{14}-14-2 to vaccine development and production, it is striking that the molecular basis for its attenuation remains largely unknown.

In seeking to understand the genetic basis for the attenuation of SA_{14}-14-2, several groups have independently determined the partial or complete nucleotide sequences of the genomes of both the SA_{14} and SA_{14}-14-2 viruses (Nitayaphan *et al*., 1990; Aihara *et al*., 1991; Ni *et al*., 1994, 1995). These comparative sequence analyses have indicated that during a series of attenuation processes, SA_{14}-14-2 acquires a large number of single point mutations, i.e., 47–64 nucleotide substitutions (17–27 amino acid changes), scattered throughout the entire viral genome (Nitayaphan *et al*., 1990; Aihara *et al*., 1991; Ni *et al*., 1995). Interestingly, there are some variations in the number of reported mutations, which are most likely dependent upon the cultivation history of the viruses used for sequencing. In the present study, we have determined the complete nucleotide sequence of the genomic RNA of SA_{14}-14-2, using viral RNA extracted directly from the commercial vaccine vials. In our hands, the genomic RNA of SA_{14}-14-2 is 10977 nucleotides long, one nucleotide longer than all the previously reported genomes of SA_{14}-14-2, SA_{14} and two other SA_{14}-derived attenuated viruses. This discrepancy is due to the insertion of one G nucleotide at position 10701 in the 3′NCR; the biological importance of this insertion needs to be tested experimentally. By comparing all available full-length genomic sequences of both SA_{14} and SA_{14}-14-2 (three different versions for each virus), we have now identified a set of 39 nucleotide substitutions (16 amino acid changes) that differ between the genomes of SA_{14} and SA_{14}-14-2. This SA_{14}/SA_{14}-14-2 system will provide us a unique opportunity to investigate the molecular mechanisms of JEV virulence, potentially promoting the development of new and improved JEV vaccines.

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