Chapter 11

Development of Japanese Encephalitis Attenuated Live Vaccine Virus SA14-14-2 and its Charcteristics

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Additional information is available at the end of the chapter

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1. Introduction

Japanese encephalitis (JE) is the most common epidemic viral encephalitis in the world today. It is estimated that the JE virus causes at least 50,000 cases of clinical diseases each year resulting in about 10,000 deaths and 15,000 cases of long-term, neuro-psychiatric sequelae. In recent decades, outbreaks of JE have occurred in several previously non-endemic areas. Nearly 3 billion people live in JE-endemic regions, where more than 70 million children are born each year. For many years, only an inactivated JE vaccine made from infected mouse brain was licensed for use by residents and travelers. However, this vaccine proved to have an unacceptable levels of adverse safety events. Recently a safe and efficacious single-dose, live-attenuated vaccine (SA14-14-2) produced in China has become available to many Asian countries. It was higher immunogenicity, fewer doses of vaccination, less side reaction and cheaper than that of the world wide used mouse brain inactivated vaccine. Since it was licensed in 1989, the vaccine has been used in more than 300 million children with no vaccine-associated encephalitis case ever reported. Currently the vaccine is produced using specific pathogen free (SPF) hamster kidney cell (PHKC) in accordance with WHO technical specifications [47]. This paper reviews the development of the SA14-14-2 vaccine and its characteristics.

1.1. Development of SA14-14-2 attenuated JE live vaccine

1.1.1. History of selecting attenuated vaccine virus SA14-14-2 strain

The vaccine virus strain SA14-14-2 was derived from a wild-type Japanese encephalitis (JE) virus SA14 isolated from pool of *Culex pipiens* mosquito larvae in Xi'an, China. Attenuation was accomplished by serial passages of the SA14 virus in primary hamster kidney (PHK) cell culture at 36 - 37℃. After 100 passages in PHK cells, followed by 3 times of plaque cloning,

one clone 12-1-7 was selected from 36 plaque clones, which exhibited lower degree of virulence,LD_{50}>6.0$log_{10}TCID_{50}$ [25]. However its neuroattenuation was unstable and reverted to the virulence of parental SA14 after 1-2 mouse brain or several PHK cell passages [54]. The 12-1-7 clone was further plaque purified for 3 times and another 37 plaque clones were obtained. However those clones still showed unstable after 1-2 mouse brain passages. Then another method was performed for further attenuation. One selected virus clone (SA14-17-4) was peripherally passaged in non-neural tissues (spleen and skin) of mice followed by several times of plaque purification, which resulted in selecting an avirulent and highly stable virus clone, SA14-9-7. However, after human clinical trial, the SA14-9-7 strain showed low immunogenicity in vaccinated children (seroconversion <10%[54]). In order to promote immunogenicity, the SA14-9-7 virus was orally passaged six times in hamster, spleens harvested for two plaque purifications. One selected clone, SA14-5-3, demonstrated higher seroconversion rates in vaccinated children, 86.2% in JE endemic area[54] and 62%in JE non-endemic area[2].The SA14-5-3 strain has been licensed for vaccine production and about five million children were vaccinated. SA14-5-3 vaccine was demonstrated to be safe but low protective efficacy, 64 - 93% in humans in the clinical trial involving 400 thousand children [54].

Methods	**Names**
SA 14 virus isolated from pool of *Culex pipiens* larvae by 11 passages in mouse brain	(SA14)
100 serial passages in PHK cells, followed by three plaque purifications in PCE cells	(SA14 clone 12-1-7)
Two plaque purifications in PCE cells	(SA14 clone 17-4)
One intraperitoneal passage in mice; harvesting of spleen for one plaque purification in PCE cells	(SA14 clone 2)
Three plaque purifications in PCE cells	(SA14 clone 2-1-9)
One passage in mice, harvesting of skin and subcutaneous tissue for one plaque purification in PCE cells	(SA14 clone 9-7)
Six oral passages in hamsters; harvesting of spleens for two plaque purifications in PHK cells	(SA14 clone 5-3)
Five passages in suckling mice; harvesting of skin and subcutaneous tissue for two plaque purifications in PHK cells	(SA14 clone 14-2)*

Table 1. Attenuation history of Japanese encephalitis SA_{14}-14-2 virus strainPCE: primary chick embryo; PHK: primary hamster kidney. * The notation SA14 clone 14-2 is abbreviated to SA14-14-2

To further promote immunogenicity, the SA14-5-3 virus was serially passaged by the subcutaneous route in suckling mice, using injected site skin and local lymph nodes for the subsequent passage materials. After cloning twice in PHK cells, the SA14-14-2 clone was selected [55]. This strain was equally attenuated compared to the SA14-5-3 but more immunogenic in mice, guinea-pigs, and pigs [55]. In human trials SA14-14-2 produced seroconversion rates greater than 90% in JE non-immune subjects living in JE non-endemic region [2]. Besides,

Eckels et al. [7] adapted the SA14-14-2 virus to primary canine kidney cell cultures for 9 passages, SA14-14-2 PDK virus, which showed avirulent in mice and monkeys. However, SA14-14-2 PDK virus resulted in an unacceptably low neutralizing antibody response - 40% seroconversion rate - in childen in China [60]. The passage history is shown in Table1 and the characteristics of the various attenuated virus derivatives are shown in Table2 and 3.

Virus/clones	Neurovirulence after different mouse brain passages					
	0	1	2	3	4	5
SA14	6.5[a] (-)[b]	7.7(8.0)	-	-	-	-
SA14-12-1-7	0(5.5)	≥1.5(-)	6.33(-)	-	-	-
SA14-12-1-1	<0.0(7.5)	2.50(6.5)	-	6.00(7.00)	-	-
SA14-9-4-2	<0.0(6.5)	≤1.83(6.0)	-	7.17/6.50	-	-
SA14-17-4	0(-)	≥4.5(≥4.5)	-	-	-	-
SA14-2	0(-)	0(-)	<1.0(5.0)	1.42(6.5)	1.0(6.0)	<1.0(6.5)
SA14-2-1-9	0(4.5)	0(≥6.5)	0(5.0)	<1.0(5.5)	1.30(≥7.5)	≥5.12(7.5)
SA14-9-7	0(6.0)	0(4.0)	0(4.0)	0(5.0)	0(4.0)	0(4.5)
SA14-5-3	0(5.0)	0(5.5)	0.62(4.0)	0(4.75)	0.58(6.5)	2.21(6.5)
SA14-14-2	0(7.0)	1.0(-)	0.58(-)	0.67(-)	0.57(-)	≤2.0(-)

Table 2. Neuroattenuation and its stability (reversibility) of various derivatives of attenuated strains from SA14 virus strainsa., Intracereble (i.c.) inoculation tested in weanling mice, $log_{10}LD_{50}$/0.03ml b., $log_{10}TCID_{50}$/0.2ml - not determined

Virus/ clones	Neurovirulence[a]		Reversibility[b]	Immunogenicity[c]
	Dose (log_{10}pfu/ml)	$log_{10}LD_{50}$/ml or No.dead/ no.tested		
SA_{14}	8.78	8.50	ND	ND
SA_{14}-12-1-7	7.7[d]	1.65	+	ND
SA_{14}-9-7	6.70[d]	0/10	-	Low
SA_{14}-5-3	6.47	0/10	-	Moderate
SA_{14}-14-2	7.17	0/10	-	High
SA_{14}-14-2 PDK	6.25	0/10	ND	Moderate

Table 3. Summary of the characteristics of various derivatives of attenuated strains from SA_{14} virus strainND not determined a) i.c. inoculation tested in weanling mice b) Reversion to neurovirulence of the parental SA_{14} virus in mice model: +, neuroreversion after one suckling mice or 1-2 weanling mice i.c. passages; -, no neuroreversion after mice i.c. passages. c) Neutralizing antibody seroconversion in humans: low, <10%; moderate, 40%~60%; high, ≥90%. d) $log_{10}TCID_{50}$/ml

1.2. Clinical studies

Vaccine safety has been evaluated in several small-scale studies and in two large-scale studies in China. Studies of 588,512 children aged between 1 and 15 years inoculated with vac-

cine from one manufacturer [35] and of 60000 children given vaccine from another manufacturer [15] reported no cases of temporally associated encephalitis. The most common adverse effect associated with vaccination was fever, which was reported in less than 0.2% of vaccinated children, with lower rates for rash and other systemic symptoms.

Daily examination of 867 vaccinated children for fever (>38℃) disclosed low rates with onset distributed evenly over the 21-day observation period, without clustering as might have been expected if onset were associated with a specific incubation period. Temperature elevations were limited to a single day in most cases.

A block-randomized coherent study of 13,266 vaccinated and 12,951 nonvaccinated children followed prospectively for 30 days has shown that no cases of encephalitis or meningitis were detected in either groups, and rates of fever, allergic, respiratory symptoms were similar in the two groups [33]. Moreover no case of encephalitis associated with the live vaccine has been reported so far from the large scale vaccination in other areas of China [66] and in countries outside China [38, 4].The vaccine is well tolerated in subjects as young as 8 months.

The immunogenicity and protection efficacy in humans have been studied several times in China and outside China. Neutralizing antibody were produced in 85%~100% of non-immune subjects studied in China [2, 58, 20, 64] and 98%, 92% and 95% studied in Korea [38], Philiphines [12] and Thailand [6], respectively after a single dose of vaccination. Several efficacy trials of SA14-14-2 vaccine in China from 1988 to 1999 in 1 to 10 year-old children have consistently yielded high protection rates, above 95%,[66, 5, 43]. One study in Guizhou province [67] and another in Anhui province [66] have shown the protection efficacy persisted for at least 11 and 5 years respectively following an immunization schedule of one primary dose at one age and one booster dose at two ages. Case-control study for evaluation the efficacy of SA14-14-2 vaccine has been studied. A case control study conducted in 1993 in Sichuan province, China in children <15 years measured vaccine effectiveness of routinely delivered SA14-14-2 vaccine at 80% for a single-dose and 97.5% for a two-dose given at a one year interval [14]. In 1999, the SA14-14-2 vaccine was given as a single dose to over 220000 residents of the Terai region of Nepal in an effort to reduce the impact of an emerging epidemic of JE. A case-control study demonstrated 99.12% efficacy [4] followed by a 5-year efficacy of 96% [41]. In 2000, a case-control study in Chongqing city, China found a 93% efficacy after one dose vaccination [42]. Besides, Kumar [23] reported a case control study in India, where 9.3 million children were immunized with SA14-14-2 vaccine in 2007, demonstrated a 94.5% vaccine efficacy after a single dose.

2. Phenotypic characteristic

2.1. *In vitro* phenotypic characteristics

SA14-14-2 attenuated virus replicates well in primary hamster kidney (PHK) cell, C6/36 mosquito cells, continuous African green monkey kidney (Vero) cells, Rhesus monkey kidney (LLC-MK_2) cells and baby hamster kidney (BHK21) cell lines. SA14-14-2 virus showed homogeneous small plaques (≤1mm) when grown in above - mentioned cells, while SA14 wild strain showed heterogeneous and larger plagues (2-3mm) [57][1].

Many attenuated viruses are temperature-sensitive, often showing restricted growth in vitro at 39-40℃, some strains were even sensitive to 37°C. SA14-14-2 strain was not temperature-sensitive, showing no reduction in infectivity at 37°C or 40°C. SA14-14-2 strain was also thermostable as the parental SA14 virus. In liquid status reduction of virus titer was 3.5log_{10}TCID50 after heated at 50℃ for 50 minutes and virus could be detected 4 hours after further incubation at 50°C, a result similar to that observed with SA14 parent strain. SA14-14-2 was more thermostabe than the SA14-5-3 [57].

2.2. Ability of *in vivo* virus replication

Yu et al. [55] studied the replication ability of SA14-14-2 virus in young mice (2.5 weeks) by subcutaneous（s.c.） inoculation followed by recovering virus from spleens and subcutaneous tissues of the infected mice for 2 weeks post inoculation. In those mice infected with the parent SA14 strain, viruses were recovered from the both tissues from day 4 to day 10 post infections, while those mice inoculated with SA14-14-2 strain, viruses were isolated in both tissues as well but limited within a short period from day 2 to day 4 or day 6 post infections (Table 4). Wu et al. [49] performed a similar study for recovering virus from brains and sera of the infected mice. Mice that infected with as less as 2.7log_{10} pfu/mL of the parent SA14 virus, high titers (≥6.0log_{10} pfu/mL) of virus was detected in the brains and low titers (2.7 – 3.4log_{10} pfu/mL) in the sera, whereas mice inoculated with as high as 6.2log_{10} pfu/mL of the SA14-14-2 ， , virus was neither detected in the brains nor in the sera of the mice. Lee et al. [24] examined virus growth of SA14-14-2 live virus and the parent SA14 virus strains in mice after i.p. inoculation. The results showed that SA14 virus was detected in sera and in spleens with peak titers of 3.24log PFU/ml and 4.3 logPFU/g, respectively as well as brains with peak titer of 6.42logPFU/g. On the other hand, SA14-14-2 virus was detected only in the spleens with extremely low titer (1.7logPFU/ml) but not detected in the sera and brains of all ten inoculated mice.

Virus strain	Infected virus titer (log_{10} $TCID_{50}$/mL)	Tissues detected	Virus titers ($log_{10}TCID_{50}$/mL) by days after inoculation					
			2	4	6	7	10	14-15
SA14-14-2	6.7	SC a tissue	3.2	4.2	1.0	ND	ND	ND
		spleen	4.2	4.2	0	ND	ND	ND
SA14	5.7	SC tissue	ND	2.7	ND	≥4.2	0	0
		spleen	ND	2.2	ND	≥4.2	≥3.2	0

Table 4. *In vivo* replication of SA14-14-2 strain in mice. a Subcutaneous tissue

Further investigation for viremia induction of SA14-14-2 virus using guinea pigs animal model has been studied by Liu et al [31]. Guinea pigs intraperitoneally (i.p.) injected with 4.0log_{10} pfu/mL of parent virus SA14 and other 4 JEV virulent strains induced viremia to virus titers of 1.0-3.0log_{10} pfu/mL and lasted for 3 days post infection. However, the animals inoculated with SA14-14-2 strain containing virus titer equal to that of the virulent strains, no viremia was detected from day 1 to day 10 post inoculation (Table 5)

Besides, a viremia clinical study in India has demonstrated the absence of any viremia activity in adult population up to 15 days after administration of a single dose of the live JE SA14-14-2 vaccine.

Virus strain	Animal No.	Viremia titer by day after inoculation (log_{10} pfu/mL)				
		1	3	5	7	10
P3	No.1	2.04	2.18	0	ND	ND
	No.2	2.08	2.59	0	ND	ND
	No.3	2.23	2.56	0	ND	ND
	No.4	1.70	2.21	0	ND	ND
02-41	No.1	1.00	1.78	0	0	0
	No.2	1.78	1.70	0	0	0
	No.3	1.78	1.60	0	0	0
	No.4	1.00	1.90	0	0	0
	No.5	1.30	1.95	0	ND	ND
HLJ02-144	No.1	1.90	1.30	0	0	0
	No.2	2.00	1.00	0	0	0
	No.3	1.95	1.48	0	0	0
	No.4	1.95	1.48	0	0	0
	No.5	2.48	ND	ND	ND	ND
SA14	No.1	2.92	ND	ND	ND	ND
	No.2	2.41	2.85	0	0	0
	No.3	2.94	3.40	0	0	0
	No.4	2.53	3.00	0	0	0
	No.5	3.02	ND	ND	ND	ND
SA14-14-2	No.1	0	0	0	0	0
	No.2	0	0	0	0	0
	No.3	0	0	0	0	0
	No.4	0	0	0	0	0
	No.5	0	0	0	0	0

Table 5. Viremia in guinea pigs after i.p. inoculation with different JEV virus strains. ND, Not determined

These results indicated that growth of the attenuated live virus in vivo was significantly reduced in contrast to the growth of parent SA14 virus, and SA14-14-2 strain showed a lack of viremia and neuroinvasion.

2.3. Neuroattenuation phenotype

Mice and rhesus monkeys are highly susceptible to the wild virulent Japanese encephalitis virus (JEV) inoculated by intracerebral route. Approximately 1~10 plaque forming unit (pfu)

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of the virus inoculated intracerebrally results in death. Mice are more susceptible than monkey to JE virus following inoculation by the peripheral route. Rhesus monkeys show four grades of response to the different attenuated JE viruses by i.c. inoculation [68]:

1. "death", the inoculation with parental SA14 virus;
2. "survival but showing neurological signs", i.e. the inoculation of SA14 95th PHK cell passage virus (SA14 HKC-95) ;
3. "survival, without showing neurological symptoms, but with fever" i.e. the inoculation with SA14-12-1-7 virus ;
4. "healthy", no fever, no symptom, no death, i.e. when the vaccine virus SA14-14-2 was inoculated.

Neurovirulence of JE SA14-14-2 strain was tested using these animal models with virus titers of 7.0~8.0log$_{10}$ pfu/ml. Weanling mice inoculated with the virus by i.c. or s.c. inoculation did not cause death. SA14-14-2 was tested by standard intrathalamic and intraspinal combination inoculation method in monkeys. Monkeys showed no mortality or morbidity and only a minimal degree of CNS inflammation around the injection sites [30] (Table 6). Further, neuropathogenicity was tested in immune- deficient or immune-suppressed animals, athymic nude mice or mice treated with cyclophosphamide. No deaths or histopathologic abnormalities were observed after intraperitoneal or subcutaneous inoculation of a viral dose greater than $10^{7.0}$ TCID50/ml. Although cyclophosphamide increases susceptibility of mice to virulent JE strains, immunosuppression with cyclophosphamide did not lead to encephalitis in mice inoculated pheripherally with SA14-14-2 virus [56, 18]. The strain also did not kill weanling hamsters by i.c. inoculations [55].

Virus strain (Virus titer, pfu/ml)	Inoculation route	Mice			Rhesus Monkeys	
		Dilution	Died/tested	Histopathological score (neuronal lesions)[a]	Died/tested	Histopathological score (neuronal lesions)[ab]
SA14 parent (6.15×10^8)	IC	10-1	ND	ND	2/2	2-4
		10-4	8/8	2-4	0/1	2-3
		10-5	ND	ND	2/2	2-4
		10-6	8/8	2-3	2/2	2-4
		10-7	8/8	2-4	2/2	2-4
		10-8	8/8	2-4	ND	ND
	SC	10-1	30/30	2-4(day 5)	ND	ND
SA14-14-2(8×10^6)	IC	1:5	0/30	0-2	0/4	0-1
	SC	1:5	0/30	0(1)[c]	ND	ND

Table 6. Comparative neurovirulence of attenuated SA14-14-2 and parent SA14 Japanese encephalitis viruses in 3-week-old mice and adult rhesus monkeysIC, intracerebral; SC, subcutaneous. ND, Not determineda) 0, No lesion; 1, ≤5%; 2, 6-20%; 3, 21-50%; 4, >50% of neurons died.b) Inoculation in thalami bilaterally (each 0.5ml) and lumbar spinal cord (0.2mL).c) One mouse showed a few dead nerve cells.

2.4. phenotypic stabilities

2.4.1. Stability of plaque morphology

Small homogeneous plaque (≤1mm) morphology was retained through 8 -17 PHK cell passages studied by Jia et al. [17]. As reported by Aihara, plaque-size phenotypes did not change during plaque purification in BHK_{21} cells and propagation in C6/36 cell [1]. Eckels et al. [7] showed that SA14-14-2 strain had a homogeneous small plaque morphology, with no large plaques seen when passed 7 times in LLC-MK_2 cells.

2.4.2. Stability of neuroattenuation

Wang et al. [44] studied neuroattenuation stability by serial passages the SA14-14-2 strain from passage 8 to passage 23 in PHK cells, virulence was tested every 2-3 passages by intracerebral or subcutaneous inoculation in mice (12 ~ 14g). None of the PHK-passaged viruses containing virus doses of 8.0~9.0 $\log_{10}$TCID50 caused death in these mice. Jia et al. [16] passed the SA14-14-2 virus in PHK cells for 17 passages, neurovirulence and neuroinvasion were determined at the 8^{th}, 15^{th}, 17^{th} passage. No animal showed illness at any the passage level.

Eckels et al. [7] performed the neuroattenuation stability by serial passage the SA14-14-2 virus in Beagle canine kidney cells for a total of 15 passages, the passage 15 virus was attenuated for young mice causing no symptoms or death by i.c. inoculation. Wang et al. [45] passaged the SA14-14-2 HKC5 virus in primary dog fetal kidney cell cultures for 11 passages. Each of the passaged virus was tested for its pathogenicity in weanling mice, all the mice survived by either i.c. or s.c. inoculation with a virus dose containing 7.5-8.0 $\log_{10}$TCID50/0.2ml. These results showed that over many passages of the SA14-14-2 virus in PHK cells or primary dog kidney cells, neuroviruence reversed to the virulence of parental SA14 was not observed.

Wu et al [49] studied on the stability of SA14-14-2 vaccine seed virus by i.c. passage in suckling mice, viruses recovered from the mice brain of passage 1 only caused a few weanling mice death following i.c. inoculation with a high dose of 8.7 $\log_{10}$pfu/ml virus (LD50≥7.7 $\log_{10}$pfu), showing no reversion to the virulence of SA14 parent virus (i.c.LD50≥0.28 $\log_{10}$pfu).

Athymic nude mice (nu/nu) inoculated intra-peritoneally with 8.2$\log_{10}$TCID50/ml of SA14-14-2 virus did not fall ill. Attempts to recover virus from brain, liver, spleen, kidney, heart and lymph nodes were made over a period of 3 weeks. In three independent experiments viruses were recovered only in one experiment from brain at the 10^{th}, 14^{th} and 21^{st} day and kidney tissue at 6^{th} day. Virus isolated from the brains on day 21^{st} after intraperitoneal inoculation, was enhanced once in PHK cell to a virus titers of 6.7-7.2 $\log_{10}TCID_{50}$/ml and then tested for neurovirulence and neuroinvasiveness in normal mice. Tests were repeated three times and showed that the recovered viruses were avirulent to 10-12g mice by i.c. or intraperitoneal inoculation, maintaining the attenuated phenotype as SA14-14-2 [56].

Neuroattenuation after long-term cold storage has been studied. Fourteen lots of lyophilized live JE vaccines manufactured year by year since 1987 were stored at low temperature (-20℃). After 15 years, neurovirulence of the vaccine viruses were studied in year 2002. The results showed that viruses in the all 14 lots were avirulent for i.c. inoculated mice. [22]

2.5. Growth characteristics and ability of transmission in mosquitoes

Mosquito infection and transmission with SA14-14-2 virus have been done using *Culex tritaeniorhynchus* mosquitoes, the most important JEV vector species, by oral feeding with meals containing the virus or intrathoracial(IT) inoculation with the virus. The mosquitoes did not become infected by oral feeding with meals containing 6.06$\log_{10}$pfu/ml virus and only one of the 34 groups (3.13%) of the infected mosquitoes became infected after feeding with meals containing 6.18 $\log_{10}$pfu/ml virus, reaching a low virus titer of 1.24$\log_{10}$pfu/ml. However, most of the mosquitoes (10 of 14 groups, 71.43%) became infected after feeding meals containing 7.85$\log_{10}$pfu/ml of virulent JE virus strain Nakayama(Nak), reaching higher titers of 3.33-4.79 $\log_{10}$pfu/ml (Table 7) [62]. The result indicated that SA14-14-2 virus is restricted in its ability to infect and replicate in the *Culex tritaeniorhychus* mosquito vector.

Virus strain	Meals containing virus($\log_{10}$pfu/ml)	No. groups tested	Total number of mosquitoes tested	No. group Positive (%)
SA14-14-2	6.06	15(4-31)[1]	345(15-36)[2]	0(0%)
	6.18	34(2-11)	573(10-39)	1(3.13%)(1.24)[3]
Nakayama	7.85	14(3-11)	215(11-26)	10(71.43%)(3.33-4.79)

Table 7. Growth of SA14-14-2 virus and wild virulent JEV Nak strain in *Culex tritaeniorhynchus* mosquitoes by oral infections.

Mosquitoes were exposed to virus-containing meals for oral ingestion. Fully engorged mosquitoes were then collected after a period of extrinsic incubation.

1. Times(days) of extrinsic incubation periods;
2. Numbers of mosquito per group;
3. Virus titers in the mosquito suspensions of the positive groups ($\log_{10}$pfu/ml)

However, virus could replicate at low level by intrathoracical (I.T.) inoculation of the *Cx.tritaeniorhynchus* mosquitoes [62], reaching titers of 2.0~3.72 $\log_{10}$pfu/ml over 2~20 days after inoculation. In contrast, mosquitoes IT infected with its parent SA14 virus exhibited higher ability of replication, reaching titers of 3.0~4.85 $\log_{10}$pfu/ml over the same periods [10].

The ability of transmission by the IT infected mosquitoes was studied later. Two groups of the mosquitoes were infected IT with SA14-14-2 virus, 8 days after infection one group of infected mosquitoes was used to infect suckling mice by direct bite, another group of infected mosquitoes was made in a suspension (M-1), in which the virus content was measured,

and used to infect weanling mice by i.c. inoculation. In order to enhance the virus titer of the mosquito suspension (M-1), it was passed once in BHK cells (M-1 C-1) and then infected mice by i.c. and s.c. inoculation. The full E protein gene of the M-1 C-1 virus was sequenced and compared to that of its parent SA14-14-2 virus. As shown in Table 8, none of the mice died after bitten or i.c. inoculation with virus titers of 4.2 and 7.2 $\log_{10}$pfu/ml, and only one nucleotide in the virus E protein gene changed resulting in one amino acid substitution (E447 A→G) which was not reverse mutation. And the eight critical amino acids remained unchanged. The similarity of the virus full E gene sequence compared to that of the parent SA14 was 99.9% [34]. This result demonstrated that the SA14-14-2 virus is phenotypic and genetic stable and could not be transmitted after mosquito passage.

Virus[a]	Virus titer	Virulence tested in Suckling mice 2.5 weeks mice				E gene sequence		
	Pfu/ml	bitten[b]	ic	ic	sc	mutation		similarity
SA_{14}-14-2 M-1	$10^{4.2}$	0/16[c]	0/16					
M-1C-1	$10^{7.2}$			0/10	0/10	0/8[d]	E-447 (A→G)	99.9%

Table 8. Virulence and E gene sequence of the SA_{14}-14-2 virus after *Culex tritaeniorhynchus* mosquitoes IT passage. a One intrathoracical passage (SA_{14}-14-2 M-1) and one BHK-21 cell passage (M-1 C-1).b By the infected mosquitoes c No. dead /no. testedd No. reversion/no. attenuating amino acidSC Subcutaneously

3. Genotypic characteristics

3.1. Gene sequence of SA14-14-2 compared to its parent SA14 and other attenuated derivatives.

When the full-length gene sequence of SA_{14}-14-2 was compared to parental $SA_{14,}$ 57-66 nucleotide substitutions were found to be scattered all over the genome except prM. These coded for 24-31 amino acid substitutions, of which 8 were in E protein[1, 61] and were studied to be the critical amino acid mutations involved in virus attenuation [3, 13] (Table 9). Among the 8 substituted amino acids observed in SA14-14-2 virus, only 3 substitutions appeared in the unstable virus SA14-12-1-7; while those highly and stable strains, SA_{14}-9-7, SA_{14}-5-3 and SA_{14}-14-2 PDK viruses had 6 changes of the 8 amino acids [Ni H et al.,1994, 8]. Two of the 8 amino acid substitutions at position E-177 (T,Threonine→A,Alanine) and E-264 (Q, Glutamine→H, Histudine) were unique to SA_{14}-14-2 virus [8] (Table 10). The contribution of these two amino acid changes to the biological properties of SA_{14}-14-2 virus requires further study.

Position		SA14-14-2		SA14
aa	nt	Zeng	Aihara	
C-65	292	S	S	L
E-107	1296	F	F	L
E-138	1389	K	K	E
E-176	1503	V	V	I
E-177	1506	A	A	T
E-264	1769	H	H	Q
E-279	1813	M	M	K
E-315	1921	V	V	A
E-334	1977	P	S	S
E-439	2293	R	R	K
NS1-292	3351	S	S	G
NS1-339	3493	M	M	R
NS1-351	3528	H	H	D
NS1-354	3539	K	K	N
NS1-392	3652	V	V	A
NS2B-63	4403	D	D	E
NS2B-65	4408	G	G	D
NS2B-87	4475	F	L	L
NS3-59	4782	V	V	M
NS3-73	4825	K	K	R
NS3-105	4921	G	G	A
NS3-343	5634	R	W	R
NS4A-27	6634	I	T	I
NS4B-106	7227	V	V	I
NS5-31	7768	G	A	A
NS5-45	7809	S	R	R
NS5-195	8261	I	M	M
NS5-386	8832	Y	Y	H
NS5-636	9593	H	Q	Q
NS5-671	9688	A	A	V
NS5-731	9898	G	D	D
NS5-759	9954	P	A	A
NS5-767	9978	V	L	L

Table 9. Comparison of amino acid differences between JE attenuated vaccine SA14-14-2 and its parent SA14 strain reported by Aihare and Zeng

Sequences were reported by [1] and [61]

3.2. Stability of gene sequence of SA14-14-2 virus strain

SA14-14-2 virus at PHK cells passage 8 (PHK_8) was serially passed to PHK17 or given one i.c. passage in suckling mouse (HKC_8SM_1). The E protein gene of the viruses was sequenced and compared to that of SA14-14-2 PHK_8 and parental SA_{14}. At passage 17, all the 8 attenuating amino acid residues in SA14-14-2 PHK_8 were retained no change, while two new nucleotide mutations were found at NT-1970 (T ▪ G), and NT-2169 (A ▪ G), which resulted in two amino acid changes at positions E-331 (S ▪ R) and E-398 (K ▪ E)(Table 11). The two substituted amino acids, Arg(R) and Glu(E), were not the residues of parental SA14, Ser(S) and Lys(K), this suggests that the two changes were not reverse mutation [9]. After one passage of the PHK_8 virus in suckling mouse brain (SA14-14-2 HK_8SM_1), 7 of the 8 amino acids remained unchanged, one at E107 (F→L) was reverse mutation, while 3 other amino acid mutations appeared at E-83, E-318 and E-327, which were not reverse mutations (Table 11).

Virus	Mutation sites							
	107	138	176	177	264	279	315	439
SA_{14}-14-2 PHK	F	K	V	A	H	M	V	R
SA_{14}-12-1-7	F	E	V	T	Q	K	A	R
SA_{14}-9-7	F	K	V	T	Q	M	V	R
SA_{14}-5-3	F	K	V	T	Q	M	V	R
SA_{14}-14-2 PDK	F	K	V	T	Q	M	V	R
Parental SA_{14}	L	E	I	T	Q	K	A	K

Table 10. Comparison of the amino acid differences in the E protein gene of JE SA_{14}-14-2 PHK vaccine virus with the other attenuated derivatives and the parental SA14

Site	SA_{14} V_2	SA14-14-2		
		HKC_8V_2	$HKC_{17}V_2$	HKC_8SM_1
E-83	E	E	E	Q
E-107	L	F	F	L
E-138	E	K	K	K
E-176	I	V	V	V
E-177	T	A	A	A
E-264	Q	H	H	H
E-279	K	M	M	M
E-315	A	V	V	V
E-318	G	G	G	D
E-327	S	S	S	F
E-331	S	S	R	S
E-398	K	K	E	K
E-439	K	R	R	R

Table 11. Substitutions of amino acid of SA14-14-2 strain after passage in PHK cell or suckling mice. HKC_8V_2, HKC 8 passages, vero cells 2 passages ;$HKC_{17}V_2$, HKC 17 passages, vero cells 2 passages ;HKC_8SM_1, HKC 8 passages, suckling mice one i.c. passage

Li et al. [27] and Gao et al.[11] performed similar studies by additional passage of the SA14-14-2 vaccine seed virus (early passage) in PHK cell culture to passage 20 and passage 18, respectively. The E protein gene sequences of the various passaged viruses were sequenced and compared to the original seed virus.The results demonstrated no reverse mutation of the 8 attenuating amino acids.

Xu et al [52] studied full-length sequence stability of the SA14-14-2 virus by passing the early passages seed virus(PHK_8) on PHK cells to passage 22 ($PHKC_{22}$) and its full-length genome was sequenced. By comparing the full sequences of the $PHKC_{22}$ with the original primary seed virus of SA14-14-2 in Genbank (D90195), the result showed that there were only 8 nucleotide differences (one in E, 5 in non-structure-region and 2 in 3′-NTR) leading to 4 amino acids changed, which were not reverse mutations. The homology of the nucleotides and amino acids between the viruses of passage 22 viruses and the primary seed virus in Genbank was 99.93% and 99.88%, respectively (Table 12). These results demonstrated that the genotype of SA14-14-2 vaccine virus was very stable during multiple cell culture passages.

Position		Nucleotide change	Amino acid change
Nt	Aa		
2142	E-389	a→c	D→N
3929	NS2a-69	t→c	none
5634	NS3-343	t→a	W→R
6634	NS4a-57	c→t	T→I
7655	NS4b-130	g→t	none
9593	NS5-639	g→t	Q→H
10701(3′-NCR)		-→g	
10784(3′-NCR)		t→c	

Table 12. Nucleotide and amino acid changes in the PHK cells passage 22 compared to the sequence of SA14-14-2 in Genbank (D90195)

4. Immunogenicity

4.1. Humoral immune response

Wills et al. [48] investigated the ability of SA14-14-2 (PHK) and other 4 attenuated vaccine clones SA14-2-8, SA14-5-3, SA14-14-2 (PDK) to induce a humoral immune response in Balb/c mice. The mice, 6-8 weeks old, were inoculated by intraperitoneal route with 10^3 and 10^6 pfu of the live viruses. Mice were bled 14 and 28 days postinoculation. Anti-JE serum antibody levels were measured using hemagglutination inhibition (HAI) and neutralization (N) tests. The results demonstrated that the live SA14-14-2 (PHK) elicited good HAI and N responses at dose of 10^3 and 10^6 pfu at 14 days postinoculation, with the 28-days sera showing no reduction in N titer. In comparison, 10^6 pfu of the SA14-14-2 (PDK) virus evoked only a poor N response by 14 days postinoculation and neutralizing antibody was not detectable with a

dose of 10^3 pfu either 14 or 28 days postinoculation (titre ≤20). The two early vaccine clones SA14-2-8 and SA14-5-3 both produced results very similar to SA14-14-2 (PDK) in terms of HAI and N responses at the given dose (Table 13).

Lee et al. [24] reported that mice vaccinated with one dose of SA14-14-2 virus ($4.0\log_{10}$pfu/ml) produced N antibody (titer 1:60), HAI antibody(22.2), complement fixation (CF) antibody (11.2) and were protected against a lethal JEV i.c. challenge (90% protection). Meanwhile, the anti-NS1(non-structural NS1) antibody was detected in sera of the vaccinated mice, which may also be responsible for the protection.

Virus strains	Titre at day 14 for dose (Log_{10} pfu)				Titre at day 28 for dose (Log_{10} pfu)			
	3		6		3		6	
	N	HAI	N	HAI	N	HAI	N	HAI
SA14-14-2(PHK)	320	320	640	80	320	NT	640	320
SA14-14-2(PDK)	<20	40	80	320	<20	160	80	NT
SA14-2-8	80	NT	160	NT	160	NT	160	NT
SA14-5-3	40	NT	80	NT	160	NT	160	NT

Table 13. Humoral immune responses (N and HAI) derived from Babl/c mice inoculated with attenuated vaccine strains. Note: N, neutralization titre, taken as the highest dilution of serum to neutralize 50% of plaque numbers of homologous virus;HAI, Haemagglutination inhibition titre, taken as the highest dilution of serum to inhibit 4 HA units of homologous virus;NT, not tested.

4.2. Protection efficacy

Several studies have demonstrated that SA14-14-2 live vaccine induced high and broad protection against challenge by various JEV virulent strains in mice. Wang et al. [46] performed a study comparing the protective efficacy of 3 kinds of JE vaccine, the SA14-14-2 attenuated vaccine, the PHK derived P3 inactivated Vaccine (iPHKV) and the mouse brain purified inactivated Nak vaccine (MBV) in mice by i.p. inoculation with one dose of live vaccine, or 2 doses of the 2 kinds of inactivated vaccines respectively followed by i.p. challenge with 2 virulent JEV P3 and Nak strains. The results indicated that despite levels of neutralizing antibodies (N) developed by the vaccination with live vaccine and MBV were equal at the day of pre-challenge, mice receiving the live vaccine were protected against the 2 challenging strains at higher rates than mice receiving the 2 inactivated vaccines (Table 14). Yu et al.[59] compared the immunogenicity of the SA14-14-2 live vaccine and iPHKV in mice by i.p. vaccination followed by i.p. challenge with 14 wild strains isolated from different areas and years in China. The results indicated that live vaccine induced higher and broader protection levels than that induced by the iPHKV. In another study, Jia et al. [21] demonstrated that mice s.c. inoculated with a single dose of 34 or 340 pfu/mL SA14-14-2 virus, mice were protected (80-100%) against i.p. challenge of the 22 JEV strains (11 isolated in China and the other 11 from Thailand, Vietnam, Indonesia, India, Philippines and Japan).

Vaccine	N titers at pre-challenge against		Protection against			
			P3		Nak	
	P3	Nak	i.p.	i.c.	i.p.	i.c.
SA14-14-2	20[a]	40	10/10[b]	8/10	10/10	4/10
iPHKV P3	10	5	7/10	ND	5/10	ND
MBV(Nak)	20	40	8/10	3/10	4/10	1/10
Control	ND	ND	2/10	1/10	2/10	3/14

Table 14. Protection of three kinds of JE vaccine in mice. Note: ND, Not determineda) Reciprocal of the highest dilution of serum that resulted in 50% reduction of plaque numbers.b) Number surviving challenge/number challenged i.p. intraperitoneally

No.	Challenge Virus strain	Genotype	Dose (pfu)				
			2340	234	23	Control	Challenge virus dose ($log_{10}LD_{50}$)
1	SH-53	I	10/10[a]	10/10	9/10	2/10	3.58
2	SH-101	I	10/10	10/10	10/10	2/10	3.17
3	LN02-102	I	10/10	8/10	4/10	1/10	3.84
4	SH03-127	I	10/10	8/10	7/10	1/10	3.71
5	HN04-11	I	10/10	10/10	5/10	2/10	2.75
6	SC04-17	I	10/10	9/10	10/10	2/10	4.00
7	SH05-24	I	10/10	9/10	4/10	1/10	3.77
8	02-29	III	10/10	10/10	7/10	2/10	3.50
9	02-41	III	10/10	10/10	6/10	2/10	4.50
10	HLJ02-134	III	9/9	10/10	6/10	2/10	2.88
11	HLJ02-144	III	10/10	8/10	4/10	0/10	3.00
12	DL04-06	III	10/10	9/10	9/10	2/10	3.24
13	P3	III	9/10	9/10	9/10	1/10	3.78
14	SA14	III	9/10	9/10	9/10	1/10	3.65
15	SA4	III	10/10	10/10	4/10	2/10	3.42
16	KT	III	10/10	9/10	5/10	1/10	3.31

Table 15. Protection efficacy of SA14-14-2 against intraperitoneal challenge with different heterologous JE virus strains. Mice were immunized with either 2340 pfu, 234 pfu or 23 pfu of SA14-14-2 virus;a) Number surviving challenge/number challenged.

However, since 1970's, a new genotype I of JEV has circulated in China while the genotype III JEV are still circulating in nature. In order to further categorize the degree of immunogenicity conferred by the SA14-14-2 vaccine against the both wild-type JEV genotypes (I and III) currently circulating in China. Liu et al [32] examined the protective efficacies of the SA14-14-2 live vaccine in mice by a single s.c.vaccination followed by i.p. challenge with 16 JEV isolates of the both genotype. As shown in Table 15,mice immunized with 2340 pfu of the live vaccine virus conferred an 80-100% protection rate against challenge with the 16 heterologous JE virus strains. Protection efficacy was 70-80% with vaccination dose as low as 234 pfu.

This result was consistent with previous data indicating SA14-14-2 vaccine conferred strong and broad protections against JEV challenge[59][21].

4.3. Suppression of viremia induction

As shown in Table 16, guinea-pigs immunized with a single SA14-14-2 dose of 5.87 pfu/mL virus induced low neutralizing antibody levels(<10 by PRNT) 14 days after vaccination, but viremia was significantly suppressed in all vaccinated animals after i.p. challenge with P3 virulent JE strain compared to control animals in which all developed high levels of viremia (2.0-3.54log_{10} pfu/mL) that last 4 days. Interestingly, despite the neutralizing antibody levels of the vaccinated animals at the day of challenge (day0) were low, higher levels antibody developed rapidly beginning at day 4 post challenge compared to the control animals that developed slowly with low level antibodies beginning at day 7 post challenge [19].

Vaccine	No. testing	Viremia by day after challenge					Titer of N antibody by day after challenge						
		2	3	4	5	7	0	2	3	4	5	7	14
SA14-14-2	1	0/2[a]	ND	ND	0/2	0/2	<4-4[c]	<4-4	ND	ND	256	1024	2048
	2	0/3	0/3	0/3	ND	0/3	<4-16	<4-16	<4-16	16-64	ND	128-1024	1024-2048
None (control)	1	3/3 (2.1-2.8)[b]	ND	ND	0/3	0/3	< 4	< 4	ND	ND	<4	<4-8	32-64
	2	4/4 (2.0-2.7)	4/4 (1.7-2.8)	2/2 (1.0-1.7)	ND	0/4	< 4	< 4	< 4	< 4	ND	8	128

Table 16. Viremia suppression in guinea-pigs after vaccination with SA14-14-2 vaccine followed by challenge with virulent JEV. a, No. viremia positive/ no. tested; b,Viremia titre,log_{10} pfu/mL; c, Reciprocal of the highest dilution of serum that resulted in 50% reduction of plaque numbers

4.4. Evidence of cellular immune responses

Several studies have shown evidence of cellular immunity induced by vaccination of SA14-14-2 vaccine in mice. Li et al.[28] studied the specific cytotoxic T lymphocyte (CTL) mediated immune responses in mice by vaccination with SA14-14-2 live vaccine and PHK inactivated vaccine. In the three testings, the average percentage of the specific CTL activity induced by mice vaccinated with one dose of live vaccine was higher (79.2%) than the mice immunized with twice doses of the inactivated vaccine (29.0%). Jia et al. [17] studied adoptive immunity in mice and demonstrated that mice received transfer of immune spleen cells from mice immunized with SA14-14-2 live vaccine was protected better (50% protection) than that from mice immunized with 2 doses of inactivated vaccine (10% protection).Another study was performed for examining the elicitation of cellular immunity by SA14-14-2 vaccine using an enzyme-linked immunospot (ELISPOT) assay. BALB/C mice were s.c. vaccinated with one dose of the live vaccine or 2 doses of a commercial SA14-14-2 inactivated PHK vaccine. Fourteen days after the initial immunization, mice were sacrificed and the the spleenocytes were isolated for detection of INF-◎ and IL-2 spot forming cells (SFC) by

ELISPOT assay. Serum samples were collected from the mice and pooled for detecting neutralizing antibody. Another group of immunized mice were i.p. challenged by virulent JEV P3 strains 14 days postvaccination. The results demonstrated that mice immunized with SA14-14-2 live vaccine produced more IFN-γ SFC ($89/10^6$ cells) and IL-2 SFC ($70\text{-}100/10^6$ cells) than mice immunized with the inactivated vaccine ($<10/10^6$ cells), respectively, the positive conversion rates of mice producing IFN-γ and IL-2 SFC following the vaccination with live vaccine compared to the vaccination with inactivated were significant i.e, 100% and 100% vs 20% and 40% respectively. However, neutralizing antibody levels in mice following vaccination with the both vaccines were similar, but higher protection effects were observed for the live vaccine immunized mice (100% vs 80%) (Table 17) [26]. These data suggested the protection correlated better with cellular immunity than neutralizing antibody responses following live vaccine vaccination. Moreover, Li et al. [29] investigated the interaction of SA14-14-2 virus with mouse bone marrow-derived dendritic cells (bmDCs). The results showed that the infection of bmDCs with SA14-14-2 resulted in viral replication and upregulation of bmDCs maturation marker molecules(CD40,CD80,CD83 and MHC1). The infection also stimulated the production of interferon-α(IFN-α),monocyte chemoattractant protein-1 (MCP-1/CCL2), tumor necrosis factor-α(TNF-α) and interleukin-6 (IL-6) of bmDCs. Furthermore, the SA14-14-2 infected bmDCs impaired the expansion of Foxp3+ regulatory T (Treg) cells with immunosuppressive potential, suggesting that SA14-14-2 infection induced antiviral immunity rather than immunosuppression. Taken together, the results indicated that SA14-14-2 infection caused bmDCs maturation, changed the expression profiles of several cytokines, and triggered T cell activation. This offered an insight in the immunologic mechanisms associated with the high efficacy of the SA14-14-2 vaccine.

Vaccine	Vaccinated dose (log_{10}pfu/ml)	ELISPOT assay		Protection	NAb
		IFN-r	IL-2		
SA14-14-2 PHK live	6.31	10/10a	10/10b	10/10c	40d
	3.31	10/10	10/10	9/10	40
	2.31	9/10	10/10	5/10	10
SA14-14-2 inactivated	Undiluted	2/10	4/10	8/10	40
Control	-	0/10	0/10	0/10	10

Table 17. Results of ELISPOT assay, protections and neutralizing antibody (NAb) responses in mice vaccinated with SA14-14-2 live and inactivated JE vaccines. a No. IFN-γ positive/no. tested mice; b No. IL-2 positive/no. tested mice; c No. survival/no. mice tested;d Reciprocal of the highest dilution of serum that resulted in 50% reduction of plaque numbers

Recently, Zhang et al.[65] investigated cytokine and chemokine responses in humans recipients (34 subjects) of SA14-14-2 live attenuated vaccine, the results indicated that levels of interleukin (IL-8), monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-α and MIP-1β were significantly higher in the vaccinees than in a control group. IL-6 was detectable in 64.7% of vaccinees, but was not detectable in any of the con-

trols. Therefore, IL-6, IL-8, MCP-1,MCP-1α and MIP-1β may play important roles in the immune response to JE live attenuated vaccine in the humans.

Besides Xu et al.[51] reported that mice immunized with SA14-14-2 virus non-structural NS1 protein, which expressed and purified from SA14-14-2 virus NS1 recombinant *E.coli* BL21 (DE3), were protected against a lethal JEV challenge (50-70% protection). Guinea-pigs vaccinated with the NS1 protein presented with reduced viremia following challenge with virulent JE virus (Table 18)

Tested groups	No. animals	Viremia (pfu/ml) by days postchallenge						
		0	1	3	5	7	10	14
SA14-14-2 NS1	1	0a	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	2. 5	0	0	0	0
	4	0	7. 5	20	0	0	0	0
	5	0	17. 5	145	15	0	0	0
Unimmunized controls	1	0	7. 5	> 579	208	0	0	0
	2	0	47. 5	260	0	0	0	0
	3	0	22. 5	318	230	2. 5	0	0
	4	0	7. 5	225	0	0	0	0
	5	0	0	> 750	ND	ND	ND	ND

Table 18. Viremia suppression in guinea pigs vaccinated with SA14-14-2 NS1 protein followed by challenge with virulent JEV. ND, Not determinedGuinea pigs were intraperitoneally (i.p.) vaccinated with NS1 protein, each 60 ug, at day 0 and 7 respectively. Fourteen days after the first vaccination, each vaccinated and unvaccinated animal was i.p. challenged with virulent JEV P3 strain (each 2.5ml).a No virus detected in the undiluted serum

5. Discussion

Japanese encephalitis is a neuroinvasive virus, which is destructive of neural tissue. Loss of neurovirulence is an important consideration in live JE vaccine development. It is well known that the mice and Rhesus monkeys are highly sensitive animals for testing neurovirulence of JE virus. Both were used during development of live JE vaccine. The process of derivation of the SA14-14-2 strain has demonstrated fine balances between stable neuroattenuation and immunogenicity.

The principal issue surrounding the clinical application of the SA14-14-2 live virus vaccine candidate is safety, especially absence of neurovirulence and stable even passage in brain tissue. During development of the SA14-14-2 strain, it was demonstrated that unstable attenuated virus clones i.e. SA14-12-1-7 and SA14-12-1-1 could be detected by several passages in PHK cells or one i.c. mouse passage. Using this method, SA14-14-2 vaccine virus was selected for its neuroattenuation stability. To ensure vaccine safety, absence of neuroreversion after one ic passage in suckling mice is required in the quality control for vaccine production.

Compared with other live attenuated viral strains, SA14-14-2 has the following characteristics:

1. Not temperature sensitive. During the course of attenuation the SA14 parental virus was cultured at 37°C, while other live vaccine strains were cultured and passaged at lower temperature (24℃ or 32-35°C);

2. Homogeneity. SA14-14-2 strain exhibits as homogeneous small plaques and is stable after several tissue culture passages. The virus strain has been purified by 14 times by a plaque picking technique. Many licensed live vaccines were developed without clonal purification, i.e. the yellow fever 17D vaccine or insufficient purification, i.e. polio vaccine strains. It was reported that the yellow fever 17D vaccine and polio Sabin vaccine virus particles may be heterogeneous [36, 40], as they were not or insufficient cloned. The mixtures may contain neurovirulent virus particles;

3. Highly attenuated for experimental animals: SA14-14-2 strain is avirulent following inoculation by intracerebral or subcutaneous routes in 2.5-week old mice. Monkeys inoculated by the combination of intrathalamic and intraspinal routes developed no signs or death, and on histopathological examination, exhibited minor inflammatory reaction only along the needle track. While the yellow fever vaccine strain 17D, in world-wide use for 70 years, may cause up to 10% death in monkeys while lethal dose in an i.c. mouse test is as high as $3.0\log_{10}$LD50. Also, polio vaccine viruses cause more histopathological lesions in monkeys than did SA14-14-2 virus;

4. Numerous substitutions of nucleotide and amino acid in the virus genome: mutation of virus genome is the molecular basis of attenuation. Molecular studies indicate that the SA14-14-2 vaccine strain differs from that of the virulent parental SA14 strain at 57-66 nucleotide locations resulting in 24-31 amino acid changes. The number of mutations observed is similar to the most stable Polio vaccine virus type I, with 56 nucleotide mutations resulting in 21 amino acid changes. Polio vaccine virus type III, which is unstable, has only 10 nucleotide mutations involving 2-3 amino acids. Polio virus type II, has 23 nucleotide mutations, also fewer than SA14-14-2. More than 70% Vaccine Associated Paralytic Poliomyelitis has been caused by Type III polio vaccine virus, which has the fewest amino acid changes from parental virus [40].

As for the immunogenicity of the SA14-14-2 vaccine, studies have demonstrated that protection efficacy of the live attenuated vaccine was mediated by the presence of neutralizing antibodies and by potent cell-mediated immunity as well as the NS1 protein induced immunity, which associated with the high efficacy of the SA14-14-2 vaccine. Besides anamnestic immune response will give quick rise of neutralizing antibody and provide long-term protection against JE infection among subjects who become seronegative after immunization with SA14-14-2 live vaccine [39]

Live JE vaccine (SA14-14-2) has been used in more than 300 million children since large scale production began in 1989. To date no vaccine associated JE cases has been reported in China and outside.The safety of live JE vaccine is due to a high degree of neuroattenuation and a number of stable phenotypic and genotypic characteristics. The combination of lack of viremia in the vaccinees and the absence of virus replication and dissemination in the mosquito

vector make the likelihood of transmission of JE vaccine virus and the risk of environment highly unlikely. Therefore the exceptional safety, stability, immunogenicity and long-term protective efficacy present a strong case for the expended use of this live attenuated JE vaccine in the world.

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