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Cloning and Sequencing of Complete cDNA of Japanese Encephalitis Virus YL Strain in Taiwan

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Abstract. We determined the complete nucleotide sequence of the YL strain of Japanese encephalitis virus and its amino acid sequence was deduced. Our results displayed that the genome of YL strain contained a single open reading frame of 10,296 nucleotides (nts) which was flanked by untranslated region (UTR) containing 95 bases at the 5′-end and 586 bases at the 3′-end, respectively. Comparison of sequences showed that the overall amino acid sequence and 3′ UTR of YL were similar to those of the virulent strain JaGAr01. However, some significant amino acid differences of viral envelope (E) protein were observed between YL and JaGAr01; the amino acid sequence of E protein in YL strain possessed $RGG_{(387–389)}$ tripeptide instead of $RGD_{(387–389)}$ in JaGAr01 and in other strains; and another amino acid is $K_{(138)}$ in YL, not $E_{(138)}$ found in others. These differences suggested that the YL strain impairs in viral attachment to the cell surface and loses neuroinvasiveness, and therefore this strain was used as a live attenuated vaccine.

Key words: genomic sequence, Japanese encephalitis virus, YL strain

Introduction

Japanese encephalitis is considered to be one of the most important mosquito-borne encephalitis in Asia and tropical Southeast Asia, and causes infection of the central nervous system in human and stillbirths in swine [1]. Viral transmission occurs in a zoonotic cycle, involving primarily Culex mosquitoes and swine as vectors and amplifers, respectively [1]. Vaccination of swine can prevent disease in swine and help to reduce JE infection in humans [2]. Recently, molecular biology study of this virus has provided a better way for the development of several viral vaccines [3–5]. Japanese encephalitis virus (JEV) is a member of the genus flavivirus that belongs to the family Flaviviridae. The JEV genome is a single-stranded (+) RNA, approximately 11 kb in length, and it contains three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [6–8]. The YL strain has been used widely as live vaccine for the control of animal Japanese encephalitis in Taiwan. For deeper understanding of molecular properties and safety concern of the YL strain, it is essential to clone and analyze the complete nucleotide of YL genome. In this work, the complete cDNA sequence and the gene map of YL strain were accomplished. Our results provided an overview on the molecular genetics of YL strain as well as the phylogenetic relationship between different strains of JEVs.

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Material and Methods

Virus Propagation

The JEV YL vaccine strain was a kind gift from the Formosa Biomedical Inc., Taiwan. Virus was propagated in ICR sucking mice brains for four days. Then,

brain tissues were harvested and homogenized to make a suspension. The cell debris was clarified by centrifugation, and the supernatants containing viruses were stored in −70°C freezer for further use.

RNA Extraction

Viral RNA was extracted from virus stock, using a pure RNA isolation kit (Blossom Biotechnologies, Inc., Taiwan). Briefly, 100 mg of tissue was homogenized in 1 ml solution for 3 min. Then sample was treated with 0.2 ml chloroform, and after centrifugation the aqueous layer was saved for the next isopropanol precipitation to obtain the RNA pellet. Purified RNA was dissolved in 100 µl water and the purity was examined by UV spectrophotometer at 260/280 nm.

Primers Design

The nucleotide sequences, locations and orientations of nineteen primers were summarized in Table 1. Primers were designed according to the published sequences and partial YL nucleotide sequences (GenBank accession numbers: AY027863). The last primer (JE3′R) was designed according to the 3-UTR similarity between YL and JaGAr01 strains.

Table 1. Nucleotide sequences of oligonucleotide primers used in RT–PCR

	Sequence (5′–3′)	Position
Name/F		
JEF	AGAAGTTTATCTGTGTGAAC	1–20
JE1F	CGGAAGATAACCATGGCTA	84–102
JES1F	CCAATGCTCCTTCGATAACC	1489–1508
JE2F	CCACAAAACAGTCCGTTGTC	1717–1736
JE7F	AGACAAGCGGATCCACCACC	2141–2160
JE4F	GAAAAAGCATGCTCTTTGCCCC	2833–2854
JE5F	ACTTTCCCCACAACCTCCTC	3960–3979
JE3F	AAACCCATCCCCCATAACC	5942–5960
JE6F	AGACCCTGAGCATCCATACC	8564–8583
JE9F	CCCTTCTGCTCTAACCATTTTC	9810–9831
JENDF	GACATAGACGAGGTGTAAGG	10729–10750
Name/R		
JE1R	CCAGTGTCAGCATGCACAT	2462–2480
JES1R	AAACATCAACCCAATCTGCC	4241–4259
JE4R	GTCTTCTCTCACACTACCCC	4813–4832
JE5R	AGTGGTATTCATCTCCAACCTG	6009–6030
JE2R	CCCATCTTCCGTATACCCTTTC	6707–6685
JE3NR	ACCACTCTAGATGACCCTG	10381–10400
JEEND	TCCTGTGTTCTTCCTCACC	10955–10973
JE3′R	AGATCCTGTGTTCTTCCTCACC CTACATACTTCGGCGCTCTGTGCCTA	10976–10924

F: forward primer; R: reverse primer.

RT–PCR

Procedures and conditions of reverse transcriptase–polymerase chain reaction (RT–PCR) were performed by one step RT–PCR with Titan™ One Tube RT–PCR system (Roche). The reaction used AMV reverse transcriptase for first strand synthesis and Taq DNA polymerase was used for the PCR. The reaction conditions were an initial denaturation step (94°C 3 min), amplification for 35 cycles (94°C 30 s for denaturation; 55°C, 30 s for annealing; 68°C, 1 min for extension), and a final extension of 10 min at 68°C in a thermal cycler (Perkin-Elmer). The RT–PCR products were analyzed by 1.2% agarose gel electrophoresis and the desired DNA fragments were purified by gel-extraction miniprep kit (Viogene) for the next experiment.

Cloning and DNA Sequencing

Overlapping cDNA products were eluted and cloned into the pGEM-T-easy vector (Promega). The cloning procedures were according to the manufacturer's instructions. These clones were examined with restriction enzyme digestion and agarose gel electrophoresis. Then sequences of these cDNA fragments were determined by the automated Applied Biosystems 310 DNA sequencer. Both strands of the PCR products were sequenced using primer walking. We used the Genetics Computer Group (GCG) software provided by the National Health Research Institutes (Taipei, Taiwan) to analyze our nucleotide sequences and construct the phylogenetic relationship.

Results and Discussion

Twenty RT–PCR products were purified and cloned into the pGEM-T-easy vector (Table 2). Figure 1 was a schematic representation of relative locations of cDNA clones to the viral genome. Most of these clones and their derived subclones were analyzed on both strands by automated DNA sequencer and primer walking. These cDNA fragments were determined partially overlapping each other and the sum of their lengths cover the entire genome

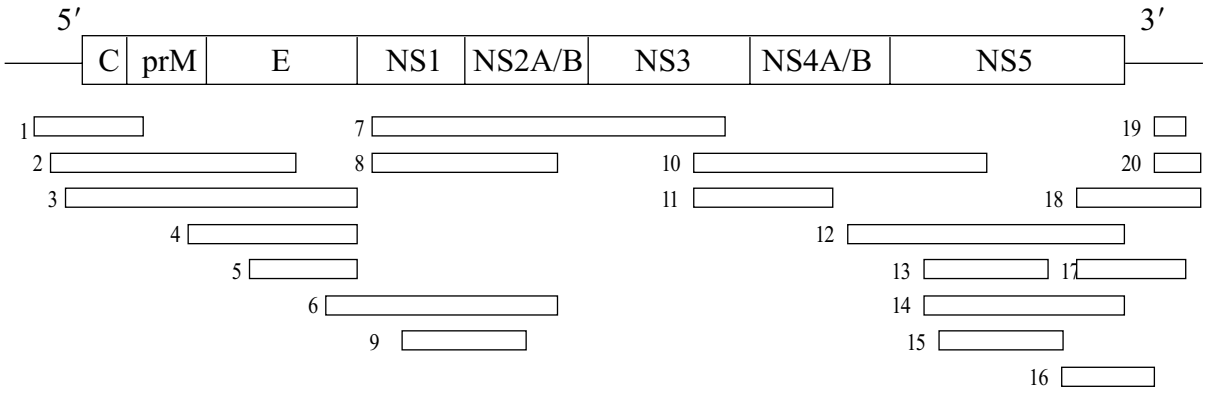

Fig. 1. The relative location of RT–PCR products to JEV genome. At the top of the figure is a genetic map of JEV genomic RNA. These empty boxes (1–20) represent RT–PCR fragments.

Table 2. Clones of cDNA products derived from the genome of YL strain

cDNAs/Clones	cDNA Location[a]	Size (bp)[b]
1. JE1	1–610	610
2. JE2	84–1883	1800
3. JE3	93–2486	2394
4. JE4	1489–2480	991
5. JE5	1919–2480	561
6. JE6	2141–4259	2114
7. JE7	2833–6030	3197
8. JE8	2833–4259	1426
9. JE9	2883–3678	795
10. JE10	5942–8989	3047
11. JE11	5942–6690	748
12. JE12	7252–10394	3142
13. JE13	8225–9049	824
14. JE14	8225–10394	2169
15. JE15	8701–9435	734
16. JE16	9434–10123	686
17. JE17	9808–10760	952
18. JE18	9810–10977	1167
19. JE19	10729–10838	110
20. JE20	10728–10977	248

[a]The location of cDNA fragments related to the YL genome sequence.
[b]The length of RT–PCR products.

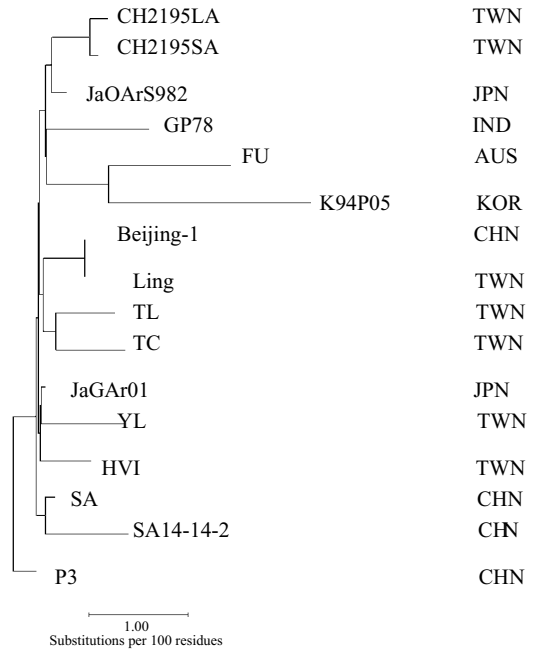

Fig. 2. Phylogenetic analysis for the full-length amino acid sequences of seventeen isolates of JEVs. GenBank accession numbers in the phylogenetic tree are: AF221499 (CH2195LA); AF221500 (CH2195SA); M18370 (JaOArS982); AF05723 (GP78); AF217620 (FU); AF04551 (K94P05); L48961 (Beijing-1); L78128 (Ling); AF098737 (TL); AF098736 (TC); AF069076 (JaGAr01); AF486638 (YL); AF098735 (HVI); D90194(SA); AF315119 (SA14-14-2); U47032 (P3). AUS = Australia; CHN = China; IND = India; JPN = Japan; KOR = Korea and TWN = Taiwan.

(10,977 nt) including the 5′ UTR (95 nt) and 3′ UTR (586 nt). Results demonstrated that the open reading frame of YL can encode a polypeptide of 3,432 amino acids with the ATG start codon at 96–98 nt and the TGA stop codon at 10,392–10,394 nt. The full genome sequence of YL strain has been submitted to the GenBank with accession number AF486638.

Construction of the phylogenetic relationship was performed using the GCG software. Comparison of the complete nucleotide sequences of YL with other 15 strains in published databases. The results showed

nucleotide similarities range from 89% to 99% and amino acid similarities from 97% to 99%. We found that the YL strain is closest to the JaGAr01, and not to the other Taiwanese isolates (such as TC, TL, HVI) or three Chinese isolates (SA, SA14-14-2 and P3) (Fig. 2). Predicted secondary structure for the 5′ UTR of YL strain displayed highly conserved features found in all other strains; in contrast, the 3′ UTR of YL was similar to that of JaGAr01, which had a very distinctive structure from nt 208 to 297 from the 3′ terminus of the viral genome (Fig. 3). YL and JaGAr01 share a unique secondary structure of the

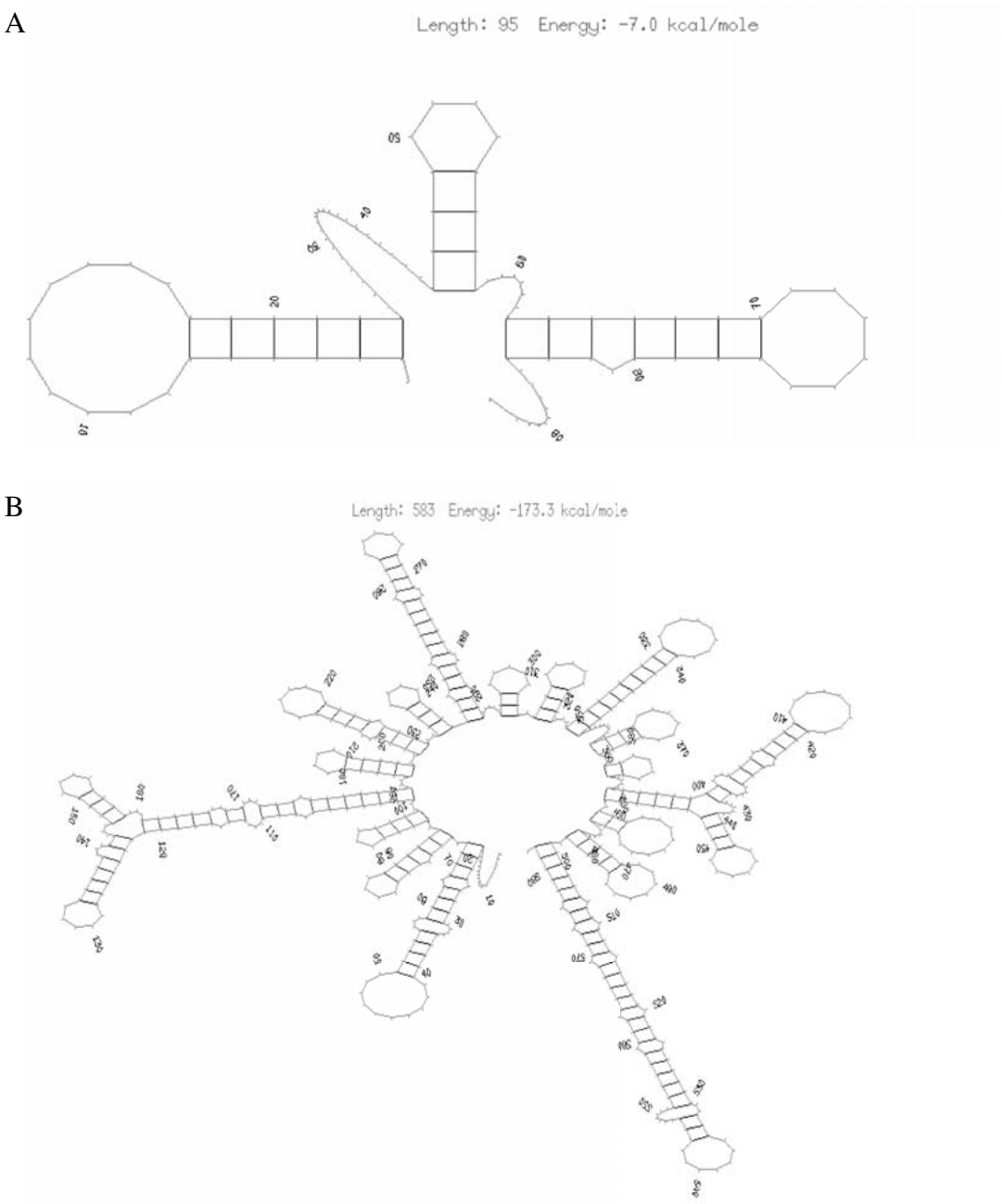

Fig. 3. Predicted secondary structures of UTRs of YL genome. (A) The 5′ UTR secondary structure. (B) The 3′ UTR secondary structure.

3′ and 5′ UTRs, suggesting that similar mechanisms are used in virus replication and virus gene expression [9–11].

Virus replication capacity in cell lines is not directly related to JEV virulence in animal [12]. It was known that amino acid changes in critical determinants of the envelope protein E are sufficient to cause diversity in virus virulence and attenuation [13,14]. In this study, we also used the E protein to construct phylogenetic tree (data not shown). For E protein, the YL strain showed the highest similarity with HVI, not with neurovirulent strain JaGAr01; and the results were compared and summarized in Table 3. The E protein of YL strain had two unique amino acids at positions 138 (K) and 389 (G). The tripeptide $RGG_{(387–389)}$ of YL strain E protein instead of $RGD_{(387–389)}$ sequence motif in other strains was related to flavivirus virulence. Another conserved amino acid $E_{(138)}$ common to other strains was replaced with $K_{(138)}$ in YL strain; this change may reduce receptor binding in central nervous system and lose of neurovirulence in YL [2,12]. The $E_{(138)}$ of JEV is a critical amino acid for E dimer formation on viral envelope to interact with cellular molecules at the stage of viral attachment [2,12]. In addition, the single-amino acid change of $E_{(138)}$ to $K_{(138)}$ was associated with small plaque phenotype [4,13]. We found the YL strain caused no plaque formation in vero cells, however viral E protein could be identified with immunostaining in infected cells (data not shown). In summary, these point mutations might influence virus propagation or interaction with host

Table 3. Amino acid differences in the E protein among strains of JEV

	S																	
aa	Beijing-1	Ling	Sa-14	JaGAr01	JaOArS982	HVI	CH2195LA	Nakayama	CH2195SA	TL	GP78	YL	TC	K94P05	FU	SA14-14-2	P3
13	E	E	E	E	E	E	E	E	E	E	E	E	Q	E	E	E	E
51	S	S	S	S	S	S	S	V	S	S	S	S	S	S	S	S	S
62	H	H	H	H	H	H	H	H	H	R	H	H	H	H	H	H	H
76	T	T	T	T	T	T	T	T	T	T	M	T	T	M	T	T	T
85	R	R	R	R	R	R	Q	R	R	R	R	R	R	R	R	R	R
102	G	G	G	G	G	G	G	G	G	G	G	G	R	G	G	G	G
107	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	F	L
108	F	F	F	F	F	F	F	F	F	F	F	F	F	F	S	F	F
132	S	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
138	E	E	E	E	E	E	E	E	E	E	E	K	E	E	E	K	E
177	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	A	T
192	P	A	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
208	S	S	S	S	S	S	S	S	S	S	S	S	S	S	P	S	S
222	A	A	A	A	A	A	A	A	A	A	A	A	A	S	S	A	A
227	S	S	S	S	S	S	S	P	S	S	S	S	S	S	S	S	P
244	E	E	G	E	E	E	E	E	E	E	E	E	E	E	E	E	E
279	K	K	K	K	K	K	K	K	K	K	K	K	K	K	K	M	K
306	E	E	E	E	E	E	E	E	G	E	E	E	E	E	E	E	G
307	K	K	K	K	K	K	K	K	K	K	K	K	K	K	N	K	K
308	F	F	F	F	F	F	F	F	F	F	F	F	F	F	S	F	F
311	A	A	A	A	A	A	A	A	A	A	A	A	A	A	R	A	A
315	A	A	V	A	A	A	A	A	A	A	A	A	A	A	V	A	A
331	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S	S
387	R	R	R	R	R	M	R	R	R	R	R	R	R	R	R	R	R
388	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
389	D	D	D	D	D	H	D	D	D	D	D	G	D	D	D	D	D
397	Y	Y	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
473	I	I	V	V	V	V	V	V	V	V	V	V	V	V	V	V	V
477	D	D	D	D	D	D	D	D	D	D	D	D	D	N	D	D	D

The E protein of YL strain showed the highest similarity with HVI. The E protein of YL strain had two unique amino acids at 138 (K) and 389 (G). The YL strain contained $RGG_{(387–389)}$ tripeptide, different from $RGD_{(387–389)}$ sequence in other strains. The $E_{(138)}$ in other strains was replaced with $K_{(138)}$ in YL strain. aa: amino acid; s: JEV strains; underline: amino acids different from other strains.

cells and provided insight into the molecular properties of the YL strain.

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References

1. Vaughn D.W. and Hoke C.H., Epidemiol Rev *14*, 197–221, 1992.
2. Lobigs M., Usha R., Nestorowicz A., Marshall I.D., Weir R.C., and Dalgarno L., Virology *176*, 587–595, 1990.
3. Mishin V.P., Cominelli F., Yamshchikov V.F., Virus Res *81*, 113–123, 2001.
4. Su C.M., Liao C.L.H., Lee Y.L., and Lin Y.L., Virology *66*, 5425–5431, 2000.
5. Zhang F., Huang Q., Ma W., Jiang S., Fan Y., and Zhang H., J Virol Meth *96*, 171–182, 2001.
6. Chamber T.J., Hahn C.S., Galler R., and Rice C.M., Annu Rev Microbiol *44*, 649–688, 1990.
7. McMinn P.C., J Gen Virol *78*, 2711–2722, 1997.
8. Rice C.M., in Fields B.N., Knipe D.M., and Howley P.M. *Fields Virology*, 3rd edn. Lippincott-Raven, Philadelphia, 1996, pp. 931–959.
9. Batista W.C., Kashima S., Marques A.C., and Figueiredo L.T., Virus Res *75*, 35–42, 2001.
10. Mangada M.N.M. and Takegami T., Virus Res *59*, 101–112, 1999.
11. Tsarev S.A., Sanders M.L., Vaughn D.W., and Innis B.L., Vaccine *286*, 206–215, 2000.
12. Wu S.C., Lian W.C., Hsu L.C., and Liau M.Y., Virus Res *51*, 173–181, 1997.
13. Chen L.K., Lin Y.L., Liao C.L., Lin C.G., Huang Y.L., and Yeh C.T., Virology *223*, 79–88, 1996.
14. Monath T.P., Arroyo J., Levenbook I., Zhang Z.X., Catalan J., Draper K., and Guirakhoo F., J Virol *76*, 1932–1943, 2002.
15. Seif S.A., Kouichi M., Sachiko M., Futoshi H., and Akira I., Vaccine *13*, 1515–1521, 1995.