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Characterization of two Japanese encephalitis virus strains isolated in Thailand

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Summary. Two strains of Japanese encephalitis (JE) virus were isolated from a pool of *Culex tritaeniorhynchus* captured in 1992 and another pool of *Cx. vishnui* captured in 1993, in Chiang Mai Area, Northern Thailand. These two strains, ThCMAr 44/92 and ThCMAr 67/93, could not be identified either as Nakayama or JaGAr01 subtype by the hemagglutination-inhibition (HI) and the neutralization (N) tests using immune sera raised against these standard JE virus strains. Reverse transcription-polymerase chain reaction showed the presence of JE-specific conserved sequences in these strains. Sequencing of 240 nucleotides in their PrM gene region identified that these two strains belong to the genotype 1 of JE virus. Nucleotide and encoded amino acid sequences of their envelope glycoprotein gene revealed 98.8 and 99.8% identity, respectively. These two strains shared 77.8 to 87.7% homology in the nucleotide sequence and 90.0 to 98.8% homology in the amino acid sequence with other reported JE strains. Five strain-specific amino acid changes were noted in ThCMAr 44/92 strain, while one in ThCMAr 67/93. In addition, four common amino acid changes were found in both strains. Thus, the findings indicated that these two strains were structurally different from each other as well as different from all the reported strains which was in agreement with the serological tests by hemagglutination-inhibition and neutralization.

Introduction

Japanese encephalitis (JE) is a serious acute disease accompanied by high fatality, and grave sequelae with neuropsychiatiric disorders among half of the survivors. Its causative agent, JE virus, is a member of the family *Flaviviridae*, genus *Flavivirus* [46, 47] and transmitted by bite of mosquitoes, such as *Culex tritaeniorhynchus*

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which breed in watered rice fields. The disease is endemo-epidemic in many countries in East, Southeast and South Asia where plenty of rice fields provide ample breeding sites for vector mosquitoes [26, 43]. In contrast to marked reduction of JE cases in Japan and in the Republic of Korea, JE epidemics became a serious health problem in several developing countries in Asia [20].

Sequence analysis of the genomic RNA of several flaviviruses has revealed that the genome is approximately 11 kb in length containing short 5′ and 3′ non-coding regions, single-stranded, positive sense in polarity, capped at the 5′-terminus and lacks a poly(A) tail at the 3′-terminus [2, 8, 10, 30, 35, 41, 48]. The flavivirus gene order has been determined as 5′-Cap-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′. The genome is translated into a single large polyprotein which is processed co- and post-translationally into three structural proteins: nucleocapsid protein (C), membrane protein (M), and envelope glycoprotein (E), as well as seven non-structural proteins: NS1 through NS5. Of the 3 structural proteins, E protein plays an important role in the biology of flavivirus. The E protein of flavivirus contain about 500 amino acids (50–60 kDa) and usually glycosylated [35, 41, 48]. The E protein of flaviviruses is viral hemagglutinin, helps the virus binding to the cellular receptor, and is capable of inducing neutralizing antibodies, which are major components in protective immunity in vivo [9, 14, 22, 29, 40]. Furthermore, it is thought that the viral envelope protein of several viruses to have a role in pathogenicity, either by determining cellular tropism [36] or by affecting virus penetration [38].

During an ecological study on JE in Thailand, 1992–1993 [45], two possible JE virus isolates were obtained from each pool of *Cx. tritaeniorhynchus* and *Cx. vishnui*, captured in Chiang Mai Area, Northern Thailand. Reverse transcription polymerase chain reaction (RT-PCR) results and nucleotide sequence analysis of their PrM protein gene confirmed that these strains belong to JE virus genotype but conventional serological tests could not identify them belonging to either of the known two subtypes of JE virus, Nakayama or JaGAr 01. Since the E protein carries the HI and N epitopes, the result suggested that some amino acid differences in the E protein should exist between these two strains and standard JE virus strains. Here we describe the antigenic characteristics of these two strains which was supported by the nucleotide and amino acid sequences of their antigenic envelope E protein gene.

Materials and methods

Mosquito specimens

Mosquitoes were collected by pig-baited net collection, once a month at 3 villages in Chiang Mai Province. These villages were coded as MT, MJ, and NT, and situated between 15–50 km from Chiang Mai City. Collection was carried out at MT from September 1992 to June 1993, at MJ from May 1992 to June 1993, and at NT from April 1992 to June 1993. After identification, collected mosquitoes were pooled according to their species, date and location of sampling as well as state of engorgement. The number of mosquitoes processed for virus isolation is shown in Table 1, which consisted of a total 183 pools.

Table 1. Number of mosquitoes processed for virus isolation

Mosquito species[b]	Collection sites and state of engorgement[a]							
	MT		MJ		NT		Total	
	f	uf	f	uf	f	uf	f	uf
Cx. tirtean	35	475	160	1400	110	1011	296	1886
Cx. vishnui	2	110	2	118	1	48	5	276
Cx. whitmor	0	5	0	0	0	0	0	5
Cx. gelidus	0	33	210	936	32	283	242	1252
Cx.. fuscoce	5	64	29	192	24	116	58	372
Total	42	687	401	2646	158	1548	601	4791

[a] *f* Engorged, *uf* unengorged

[b] *Cx. tirtean: Cx. tritaeniorhunchus, Cx. whitmor: Cx. whitmorei, Cx. fuscoce: Cx fuscocephala*

Cell culture

Aedes albopictus clone C6/36 cell line was grown at 28 °C with Eagle's medium in Earle's saline supplemented with 0.2 mM each nonessential amino acids and 9% heat-inactivated fetal calf serum [19]. BHK21 cells were grown at 37 °C with the same medium as C6/36 cells. The serum concentration was reduced to 2% in the maintenance medium.

Virus isolation

Each pool of mosquitoes was ground with 2 ml of the maintenance medium in a motor-driven glass homogenizer chilled in ice-water. The homogenate was centrifuged at 2500 rpm for 15 min at 4 °C, and passed through 0.2 μm filter. Growth medium was removed from C6/36 cell culture in rubber-stoppered 16×20 ml tubes, and 0.1 ml of the filtrate was inoculated into each tube. After 2 h adsorption, the cells were covered by 2 ml/tube of the maintenance medium and incubated at 28 °C for a week. Presence of the flavivirus antigen in the infected culture supernatant was screened by microsandwich ELISA [44]. In this test, anti-flavivirus IgG which was prepared from high-titered dengue hemorrhagic fever patient's sera was used as a catching antibody, and the same IgG conjugated with horseradish peroxidase (HRPO) as a detecting antibody, respectively. Any specimens showing more than 1.5 ELISA-OD of the negative control were further passaged in C6/36 cells to confirm the production of flavivirus antigen in the culture supernatant.

Hemagglutination-inhibition (HI) and neutralization (N) tests

Standard anti-JE rabbit sera were raised by repeated intramuscular inoculation of mouse brain grown Nakayama or JaGAr01 strain. Test virus strains were grown in C6/36 cells at 28 °C, and the infected culture fluids were stored at −70 °C in aliquots.

HI test was carried out by the standard method [7,39]. The antigens were either infected C6/36 cell culture fluids for new isolates, or sucrose-acetone extracted antigen from suckling mouse brains infected with Nakayama or JaGAr01 strain.

N test was carried out by the micromethod which was modified from Okuno et al. [33]. Constant amount of the virus in the virus diluent (cell growth medium from which serum concentration was reduced to 5%) was mixed with serially 10-fold diluted antiserum. After 1 h inoculation at 37 °C, the serum-virus mixtures were inoculated into replicate wells of BHK21

cell culture on 96-well flat-bottom plates using 50 µl/well. After 2 h adsorption, the cells were covered by 100 µl/well of the overlay medium (0.5 % methylcellulose 4000 in the maintenance medium) and incubated at 37 °C in 5% CO_2-atmosphere for 30 h. The overlay medium was removed, the cells were fixed with 5% formaldehyde for 20 min, permealized with 1% Nonidet P40 for 20 min, and successively reacted with anti-JE mouse serum at 1:500 dilution and HRPO-conjugated anti-mouse IgG at 1:1000 dilution for 1 h each. Intracellular viral antigen was revealed by HRPO reaction using substrate solution of 0.2 mg/ml 3,3′-diaminobenzidine tetrahydrochloride and 0.02% hydrogen peroxide. The number of foci consisting of brown-stained cells was counted under a magnifier lens. The percent reduction of the foci obtained for each serum dilution was plotted against the serum dilution on a probit chart, to estimate 50% focus reduction titer of the serum.

Reverse transcription-polymerase chain reaction (RT-PCR)

ELISA antigen positive culture fluid from infected C6/36 cells was first examined by flavivirus cross-reacting primer pairs (YF-1: 5′-GGTCTCCTCTAACCTCTAG-3′ and YF-3: 5′-GAGTGGATGACCACGGAAGACATGC-3′) followed by the confirmation using JE-specific primer pairs (JE-NS3-1S: 5′-AGAGCGGGAAAAAGGTCAT-3′and JE-NS3-4R: 5′-TTTCAC GCTCTTTCTACAGT-3′) as described previously [27, 42]. Briefly, 5 µl of infected fluid was incubated with an equal volume of detergent mix [1% Nonidet P-40, 10 U RNase inhibitor (Takara, Kyoto, Japan)] in phosphate-buffered saline without calcium or magnesium in a 0.5 ml Eppendorf-type centrifuge tube for 1 min at room temperature (RT). This was followed by the addition of 90 µl of RT-PCR mix [100 pmol of each primer, 0.2 mM deoxynucleotide triphosphate, 10 mM Tris (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg of bovine serum albumin per ml, 0.1% sodium cholate, 0.1% Triton X-100, 10U reverse transcriptase (Life Science Inc., U.K.) and 2U Tth DNA polymerase (Toyobo, Osaka, Japan)]. The reaction mixture was covered by two drops of mineral oil, and the tube was incubated for 10 min at 55 °C for the first strand cDNA synthesis by reverse transcriptase reaction. PCR amplification was carried out in 25 cycles, using denaturation at 94 °C for 60 sec, annealing at 55 °C for 40 sec and extension at 72 °C for 60 sec using a thermal cycler (Iwaki, Tokyo, Japan). The final chain extension step was at 72 °C for 5 min. The PCR products were detected by electrophoresing 10 µl in 3% NuSive GTG agarose (FMC Bioproducts, Rockland, USA) gels containing 0.1 µg/ml of ethidium bromide and TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 7.5).

Preparation of viral genomic RNA

Viral genomic RNA was extracted from infected culture fluid using ISOGEN reagent (Nippon Gene, Japan) following the manufacturer's instruction. Briefly, 400 µl of tissue culture supernatant was mixed with 800 µl of ISOGEN solution in a 1.5 µl Eppendorf tube and kept at RT for 5 min. Then 160 µl of chloroform was added to the mixture and vigorously shaken for 30 sec and stored at RT for 2 min. After centrifuging, aqueous phase was collected into a fresh Eppendorf tube, and precipitated with isopropanol and centrifuged to pellet the RNA. The pellet was washed with 70% ethanol, air dried and resuspended in RNase free 50 µl of sterile distilled water and stored at −80 °C until used.

Nucleotide sequencing of the PrM gene

The target sequence of JE virus PrM gene region was amplified by RT-PCR using following primer pairs: sense primer 5′-$_{414}$GGAAATGAAGGCTCAATCATGTG$_{436}$-3′ and anti-sense primer 5′-$_{739}$TTGGAATGCCTGGTCCG$_{723}$-3′ [4, 31]. For a 100 µl reaction, 5 µl of RNA preparation was added to the 95 µl RT-PCR mix and subjected for amplification for 30 cycles. Seventy microliters

of the RT-PCR product were separated from the primers and 4 dNTPs by filtration through Quick Spin Columns (Boehringer, Germany). The sequence of the recovered DNA was determined by dideoxy chain termination method [37] in an Applied Biosystems Model 373A DNA Sequencer using a Taq dye primer cycle sequencing kit (Applied Biosystems).

cDNA synthesis, cloning and nucleotide sequencing of the E protein gene

Three over-lapping regions (nucleotide numbers 960 to 1503, 1458 to 2023, and 1978 to 2501) of the two JE virus strains were amplified with primers JE960S (5′-$_{960}$GTCCGTCCGGCTTACAGTTT $_{979}$-3′) and JE1503R (5′-$_{1503}$GAAGGAGCATTGGGTGTTACT$_{1418}$-3′), primers JE1458S (5′-$_{1458}$GCGTCCCACGCGGCAAAGTT$_{1477}$-3′) and JE2023R (5′-$_{2023}$GTCATGTCATTGAGGCTCGCA$_{2003}$-3′), and primers JE1978S (5′-$_{1978}$CCTGCAAAATTCCGATTGT$_{1996}$-3′) and JE2501R (5′-$_{2501}$GATGTCAATGGCACATCCAGT$_{2481}$-3′) by direct RT-PCR using extracted RNA as described above. These oligonucleotide primers were selected on the basis of the published sequence for the JaOArS982 strain of JE virus [41]. All the primers were synthesized in an Applied Biosystems 392 DNA/RNA synthesizer and the purity of the product was confirmed by an ion-exchange gel chromatography (Gene-pack, Waters).

The PCR amplified products were separated in 1% agarose gels. Nucleic acid species with expected size were excised and eluted using Gene Clean II (BIO 101, La Jolla, CA, USA). The DNAs were then ligated directly into the TA Cloning pCR II vector using the TA cloning system (Invitrogen, USA) and used to transform competent *Escherichia coli* strain JM109 following the manufacturer's instruction. The recombinant colonies were screened by blue-white color reaction on X-gal containing plates and the cDNA inserts were confirmed by *Eco*R1 digestion and PCR.

DNA extracted from two different colonies from each independent PCR reaction was sequenced in both directions to verify the sequences. Furthermore, the nucleotide variation in certain positions was confirmed by direct sequencing of the PCR products as described above.

Data analysis

Nucleic acid sequence data were analyzed with the aid of DNASIS Software version 2.2 (Hitachi Software Engineering, Yokohama, Japan), and the secondary structure and hydrophilicity of the E protein was predicted by the Chou and Fasman, and Hoop and Woods methods, respectively [6,16]. E protein gene sequence of JE virus strains used in this analysis were obtained from following sources: JaOArS982 [41], Nakayama-RFVL [12], Beijing-1 [12], Kamiyama [12], 691004 [12] and Muar [12]. Unpublished data on JaOH0566 were obtained by our study group.

Nucleotide sequence accession numbers

The nucleotide data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence databases with the following accession numbers: PrM protein gene of ThCMAr44/92, D45360; PrM protein gene of ThCMAr67/93, D45361; E protein gene of ThCMAr44/92, D45362 and E protein gene of ThCMAr67/93, D45363.

Results

Possible JE virus isolates from mosquito pools

Out of total 183 pools inoculated into C6/36 cell culture for virus isolation, specimens from 3 pools consistently produced significant levels of flavivirus ELISA antigen in 3 successive transfers in C6/36 cell cultures. These specimens were designated as ThCMAr44/92 (abbreviated as 44/92) which was obtained from a pool

of engorged *Cx. tritaeniorhynchus* captured in April 1992 at NT, ThCMAr105/92 (abbreviated as 105/92) which was obtained from a pool of unengorged *Cx. tritaeniorhynchus* captured in August 1992 at MJ, and ThCMAr67/93 (abbreviated as 67/93) which was obtained from a pool of unengorged *Cx. vishnui* captured in June 1993 at MT.

The HI test was used for the first identification on these specimens, and the result was shown in Table 2. Two standard JE virus strains, Nakayama and JaGAr01, were antigenically distinct as reported previously [32]. The HI titers of both standard anti-JE rabbit sera were significantly less against new isolates compared with each homologous antigens. The HI titer of the anti-Nakayama serum was similar to JaGAr01 and 44/92 strains but a little less to 67/93 and a significantly less to 105/92 strain. The HI titer of the anti-JaGAr01 serum was similar to Nakayama and 44/92 strains but significantly less to 67/93 and especially to 105/92 strains. Therefore, these three new isolates cannot be assigned either to Nakayama or JaGAr01 subtype by the HI test, and 105/92 strain could most distantly be related to these standard JE virus strains.

The N test was carried out only for 44/92 and 67/93 strains because 105/92 strain did not form foci in BHK21 cells as other JE virus strains. The result in Table 2 again showed that Nakayama and JaGAr01 strains of JE virus are distinguishable by each standard antiserum. The N titer of anti-Nakayama serum was higher to 44/92 than to JaGAr01 strain, but still significantly less than the titer to the homologous Nakayama strain. The N titer of anti-JaGAr01 serum was a little lower to the 44/92 than to the Nakayama strain, but both titers were significantly less than the titer to the homologous JaGAr01 strain. The 67/93 strain was less neutralizable by both standard anti-JE sera compared with other strains. Therefore, even the N test could not assign these two new isolates to either of the known subtype of JE virus.

Identification of the isolates by PCR

Since standard identification methods by the classical serology could not provide definitive answer, molecular methods were applied to characterize possible candidates of JE virus isolates. The RT-PCR by flavivirus cross-reacting primer

Table 2. HI and N tests on newly isolated virus strains, 44/92, 67/93 and 105/92, using standard anti-JE virus sera

Virus strains	HI by		N by	
	anti-Nakayama	anti-JaGAr01	anti-Nakayama	anti-JaGAr01
Nakayama	2560	640	2.5×10^6	3.4×10^3
JaGAr01	160	2560	1.6×10^4	6.0×10^5
44/92	80	640	1.2×10^5	8.2×10^2
67/93	40	160	1.8×10^2	4.6×10^2
105/92	20	20	nt[a]	nt

[a] *nt* Not tested because 105/92 strain did not form foci in BHK21 cells

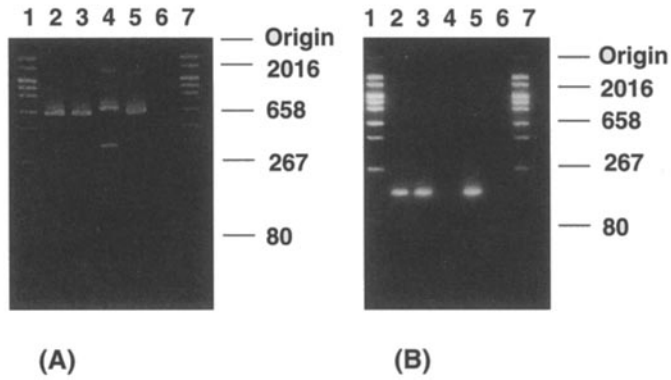

Fig. 1. Ethidium bromide stained RT-PCR products separated by agarose gel electrophoresis. **A** RT-PCR by flavivirus cross-reacting primers. **B** RT-PCR by JE-specific primers. *1* and *7* DNA molecular weight marker, *2* 44/92, *3* 67/93, *4* 105/92, *5* JaGAr01 as positive JE virus control, *6* negative control

pairs amplified all three new strains (44/92, 67/93 and 105/92), indicating the presence of conserved sequence in their 3′-terminus (Fig. 1 A). The second RT-PCR using JE-specific primer pairs could amplify only two of the three candidate strains: 44/92 and 67/93 (Fig. 1 B). Negative amplification of the remaining 105/92 strain is compatible with its biological and serological characteristics as described above (Table 2).

Nucleotide sequence analysis of PrM protein gene

For further confirmatory test and genotype determination, 240 nucleotide sequence in the PrM gene was analyzed for these two strains. Both 44/92 and 67/93 strains were found to possess identical nucleotide sequence in this region as shown in Fig. 2 in comparison with the reference JaOArS982 strain [41]. In order to find out any similarities between these two new isolates and JE virus genotypes as reported by Chen et al. [4, 5], homology comparison was made between their sequences. The results in Table 3 showed that these two new isolates possessed highest homology to other strains of the genotype 1, with nucleotide sequence homology between 91.25 to 95%, and amino acid sequence homology between 96.25 to 100%. The homology to genotypes 2 and 3 strains were similar: genotype 2 82.91–87.91% in the nucleotide and 88.75–95% in the amino acid; genotype 3 82.92–87.5% in the nucleotide and 91.25–96.25% in amino acid. By contrast homology to genotype 4 strains appeared lower, namely 82.5–85.42% in the nucleotide and 86.25–91.25 in amino acid, respectively.

Nucleotide sequences analysis of the E protein gene

Finally, the nucleotide sequence of the E protein gene of 44/92 and 67/93 strains was analyzed to reveal the possible reason associated with their unique behavior in the HI and N tests. The complete nucleotide sequences of the E protein gene of the two JE virus strains, 44/92 and 67/93, were aligned and compared with other published

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                456
JaOArS982       GUCAUAGCUUACGCAGGAGCAAUGAAGUUGUCGAAUUUCCAGGGGAAGCUUUUGAUGACCAUCAACAACACGGACAUUGCA
ThCMAr4492      --U-C---C-GU--C-----C------C-A--A--C--U--A--A------C--------------------------G
ThCMAr6793      --U-C---C-GU--C-----C------C-A--A--C--U--A--A------C--------------------------G

JaOArS982       GACGUUAUCGUGAUUCCCACCUCAAAAGGAGAGAACAGAUGCUGGGUCCGGGCAAUAGACGUCGGCUACAUGUGUGAGGAC
ThCMAr4492      -----C--------C-------------U----------U--------A-----A-----U--U----------A---
ThCMAr6793      -----C--------C-------------U----------U--------A-----A-----U--U----------A---

                                                                                          695
JaOArS982       ACUAUCACGUACGAAUGUCCUAAGCUCACCAUGGGCAAUGAUCCAGAGGAUGUGGAUUGCUGGUGUGACAACCAAGAA
ThCMAr4492      --C----------------G-----UG--G-------C-----G--A--C-----C--------C-----U------
ThCMAr6793      --C----------------G-----UG--G-------C-----G--A--C-----C--------C-----U------
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Fig. 2. Sequence of 240 nucleotides in PrM gene region of 44/92 and 67/93 strains compared with JaOArS982 as standard JE virus strain. Only nucleotide differences from JaOArS982 are shown. Nucleotide positions are numbered according to Sumiyoshi et al. [41]

strains [12, 41] as shown in Fig. 4. The nucleotide differences are scattered throughout the length of the gene, and there is no particular region of hypervariability.

The strains 44/92 and 67/93 possessed 98.8% nucleotide identity, with 18 nucleotide sequence differences each other. Of these 18 substitutions, 5 were transversional mutations at position 393, C-A; 877, C-A; 1052, G-T; 1062, C-G; and 1440, A-C. The changes at positions 393, 877, and 1052, resulted in three amino acid codon changes, whereas two others were silent mutations. The remaining 13 were transitional changes, of which 3 substitutions at position 1000, C-T; 1118, G-A; and 1475, C-T; resulted in 3 amino acid codon changes, while others were silent mutations.

The nucleotide sequences of strain 44/92 revealed 87.7 to 88.7% nucleotide identity (average divergence 11.8%) with other published JE virus strains except Muar, a strain isolated from human brain in Malaysia in 1952. The strain 67/93 also showed 87.7 to 88.7% nucleotide identity with other published strains except Muar. These two strains, 44/92 and 67/93, showed only 77.8 and 78.0% nucleotide similarity (average divergence 22.0%) with Muar strain. Thus this result indicates that these two strains are most distantly related to Muar strain whereas closely related to JaOArS982, a strain isolated from a mosquito pool in Osaka, Japan in 1982 (88.7% nucleotide identity).

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                121                                                                       201
JaOArS982       VIAYAGAMKLSNFDGKLLMTINNTDIADVIVIPTSKGENRCWVRAIDVGYMCEDTITYEGPKLTMGNDPEDVDCWCDNQE
ThCMAr4492      -T-C-------------------------------------------------------------AV--------------
ThCMAr6793      -T-C-------------------------------------------------------------AV--------------
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Fig. 3. PrM protein amino acid sequence of 44/92 and 67/93 strains deduced from their nucleotide sequence in Fig. 2 compared with JaOArS982 strain

Table 3. Nucleotide (NT) and amino acid (AA) sequence homology (%) of PrM gene region of 2 new Thai JE virus strains, 44/92 and 67/93, compared with other strains

Genotype	Strain name	Isolation		Homology %	
		country	years	NT	AA
I	M-864	Cambodia	1967	91.25	96.25
	M-859	Cambodia	1967	92.91	96.25
	B-2582	Thailand	1985	94.16	100.00
	KE-105/83	Thailand	1983	93.73	100.00
	B-0860/83	Thailand	1983	95.00	100.00
	KE-82-008	Thailand	1982	92.91	100.00
	2372/787	Thailand	1979	95.00	100.00
	2909/84	Thailand	1984	94.58	100.00
	KE-093/83	Thailand	1983	94.58	100.00
	KPO-439	Thailand	1984	94.16	100.00
	B-2239	Thailand	1984	91.66	100.00
II	JE-827	Sarawak	1968	87.50	91.25
	JKT-1724	Indonesia	1979	82.91	88.75
	B-1065/83	Thailand	1983	86.25	93.75
	B-1034/83	Thailand	1983	86.66	93.75
	WTP/70/22	Malaysia	1970	87.91	95.00
III	Nakayama	Japan	1935	87.08	95.00
	JaGAr01	Japan	1959	85.00	93.75
	JaoArS982	Japan	1982	84.60	95.00
	Beijing-1	China	1949	87.00	96.25
	B-2524	Nepal	1985	87.50	96.25
	9548	Nepal	1985	87.50	96.25
	H-49778	Sri Lanka	1987	86.66	96.25
	PhAnl242	Philippines	1984	82.92	91.25
IV	JKT-7003	Indonesia	1981	85.42	90.00
	JKT-6468	Indonesia	1981	85.42	90.00
	JKT-7887	Indonesia	1981	85.42	91.25
	JKT-8442	Indonesia	1980	82.50	86.25
	JKT-9092	Indonesia	1981	83.00	93.75

Amino acid differences in the E protein of the isolates

The deduced amino acid sequences of the E protein from JaOArS982 strain is shown, in the standard one letter code, in Fig.5, and only the differences are noted for the other strains. All the twelve cysteine residues were conserved, and the RGD motif at positions 388–390 was also conserved in these 44/92 and 67/93 strains as well as all other strains.

Comparison of the deduced amino acid sequence of 44/92 and 67/93, revealed a 98.8% conservation in the amino acid sequence, with 6 amino acid differences between these two strains. These were histidine-glutamine, glutamine-lysine, proline-serine, glycine-valine, glycine-glutamic acid, and alanine-valine at positions

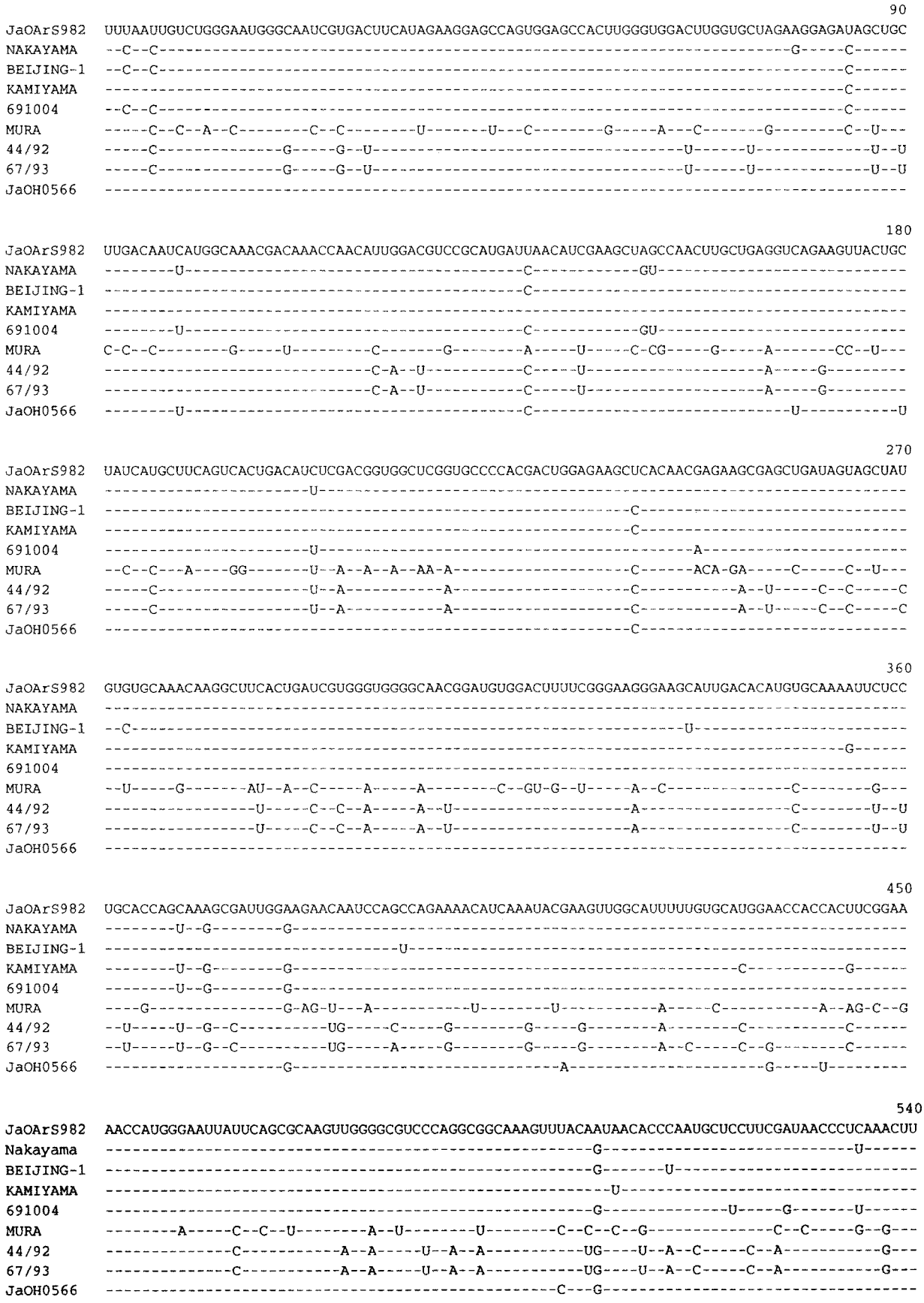

Fig. 4 (*continued*)

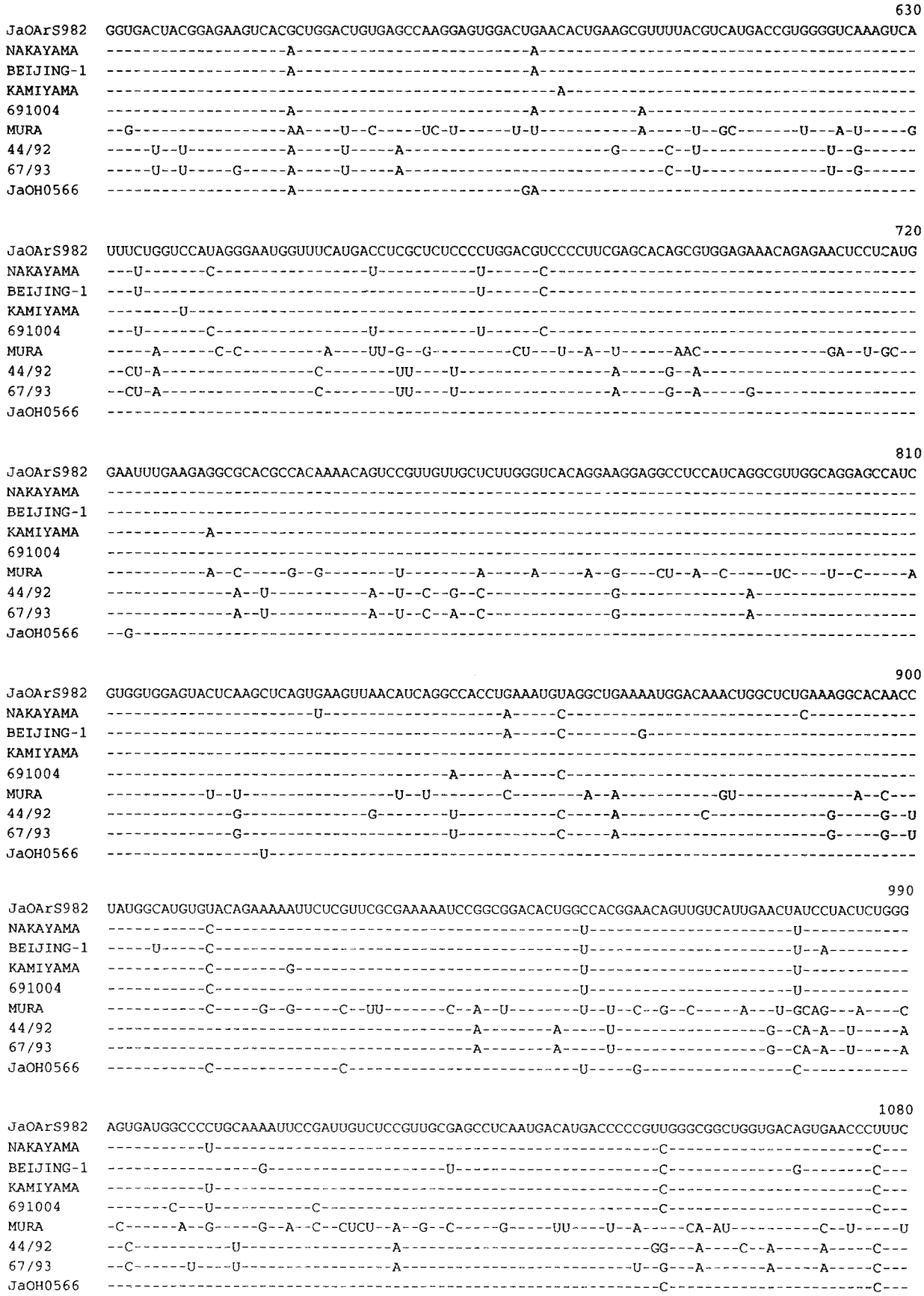

Fig. 4 (*continued*)

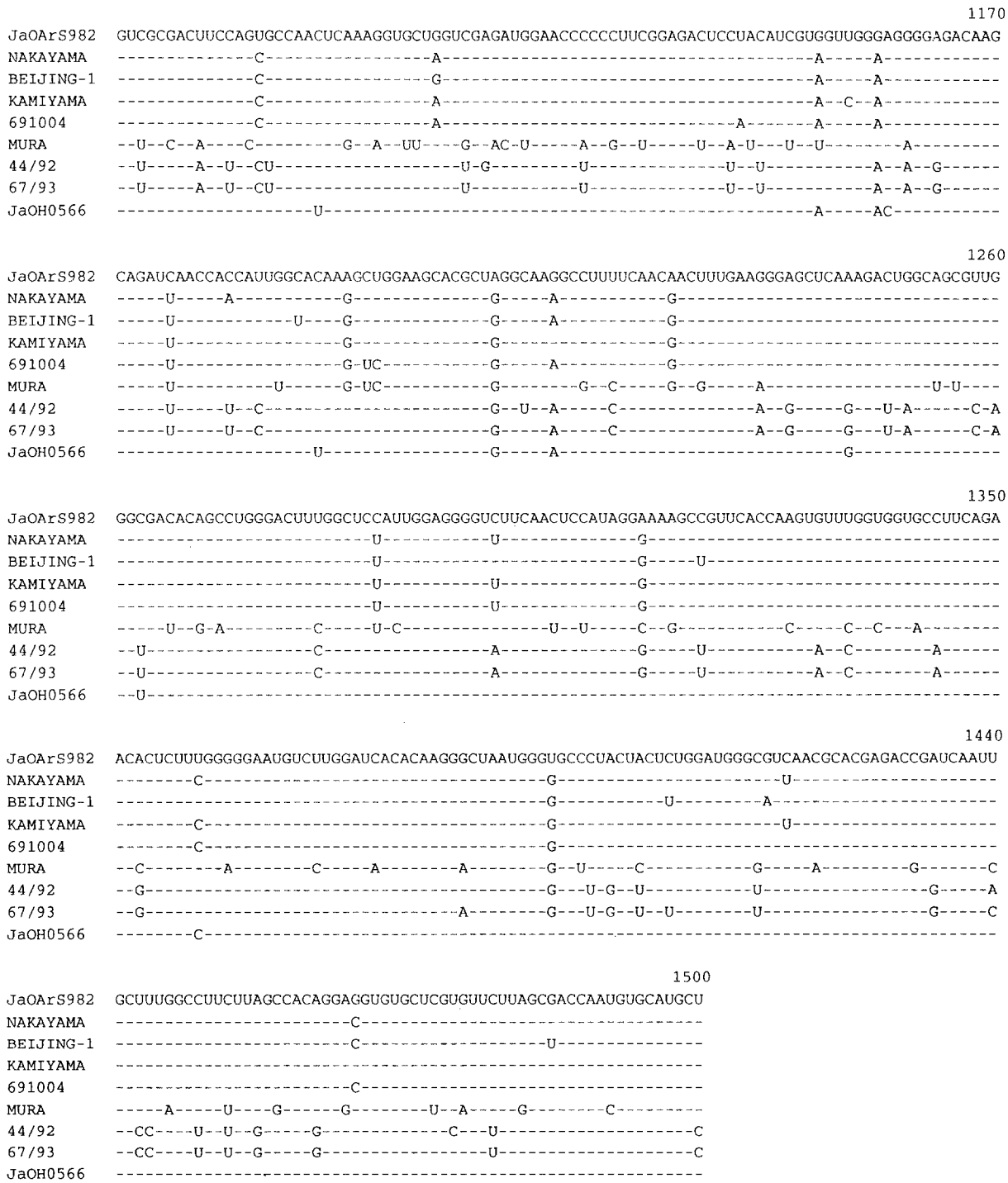

Fig. 4. Nucleotide sequences comparison of the E-protein gene of 44/92 and 67/93, and 7 JE virus strains [12, 41]. The sequence of JaOArS982 is written in full, while only changes in nucleotides are indicated for the sequences of the other strains. Nucleotides are numbered from the first nucleotide of the E coding region

Table 4. Strains specific amino acid changes in 44/92 and 67/93 JE virus strains compared with JaOArS982 strain

	Strain	Nucleotide change (position)	Amino acid change (position)
Strain specific change	44/92	CAG → CAC (393)	Gln → His (131)
		AAA → CAA (877)	Lys → Gln (293)
		CTT → GGG (1052)	Val → Gly (351)
		GAG → GGG (1118)	Glu → Gly (373)
		GTG → GCG (1475)	Val → Ala (492)
Strain specific change	67/93	CCC → TCC (1000)	Pro → Ser (334)
Change common in both strains	44/92 and 67/93	ACA → ATG (386)	Thr → Met (129)
		GCT → TCT (664)	Ala → Ser (222)
		TCC → ACA (979)	Ser → Thr (327)
		GCC → TCC (1096)	Ala → Ser (366)

Position numbers are counted from the first nucleotide of the protein E-coding region and the first amino acid of protein E of JaOArS982 strain [41], respectively

131, 293, 334, 351, 373, and 492, respectively. The strain 44/92 showed 96.6 to 98.0 % amino acid identity with other published strains except Muar, whereas 67/93 showed 97.4 to 98.8% identity. These two strains, 44/92 and 67/93, showed only 90.0 and 90.8% amino acid identity with Muar strain. Thus it again indicates that they are most distantly related to Muar, whereas very closely related to JaOArS982 strain (98.0 and 98.8% identity, respectively).

Nucleotide and deduced amino acid sequence analysis revealed that most of the nucleotide substitutions were located at the third position of the respective codons and therefore, represent transitionally silent mutations (Figs. 4 and 5). These substitutions resulted in only 9 amino acids changes in 44/92 strain, in contrast to 5 amino acid changes in 67 /93, compared with the JaOArS982 strain.

The strain 44/92 exhibited 5 strain-specific amino acid substitutions. Compared with JaOArS982 strain, these substitutions were at position 131, glutamine to histidine; 293, lysine to glutamine; 351, valine to glycine; 373, glutamic acid to glycine; and 492, valine to alanine. Four of the five substitutions caused a change in polarity and/or charge (Table 4). The substitution at position 351 resulted in a change from a hydrophobic to hydrophilic residue. Amino acid sequences around positions 131 and 351 are hydrophilic and possess a β-turn structure. Two substitutions at position 293 and 373 introduced residues which could interfere with the formation of α-helices.

The strain 67/93 showed one strain-specific amino acid change, from proline to serine at position 334, a change from hydrophobic nonpolar to hydrophilic polar residue. The amino acid sequence around this residue is hydrophobic and has a β-turn structure.

These two strains, 44/92 and 67/93, possessed 4 common amino acid substitutions: at position 129, threonine to methionine; 222, alanine to serine; 327, serine

```
                                                                                                      90
JaOArS982   FNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPTLDVRMINIEASQLAEVRSYCYHASVTDISTVARCPTTGEAHNEKRADSSY
NAKAYAMA    --------------------------------------------------V---------------------------------------
BEIJING-1   ------------------------------------------------------------------------------------------
KAMIYAMA    ------------------------------------------------------------------------------------------
691004      --------------------------------------------------V-------------------------------K-------
MUAR        --------------V-----------------------------------T------T-----T-A----------------TR------
44/92       ------------------------------------------------------------------------------------------
67/93       ------------------------------------------------------------------------------------------
JaOH0566    ------------------------------------------------------------------------------------------

                                                                                                     180
JaOArS982   VCKQGFTDRGWGNGCGLFGKGSIDTCAKFSCTSKAIGRTIQPENIKYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTITPNAPSITLKL
NAKAYAMA    -----------------------------------------------------------------------------IV-----------
BEIJING-1   -----------------------------------------S------------------------------------V-----------
KAMIYAMA    ------------------------------------------------------------------------------------------
69004       ------------------------------------------------------------------------------V----S-V----
MUAR        -----Y----------------------L--S-----KI-------------------A-------------------------------
44/92       --------------------------------------M-H-------------------------------------V-----------
67/93       --------------------------------------M---------------------------------------V-----------
JaOH0566    -----------------------------------------------K-------A----------------------V-----------

                                                                                                     270
JaOArS982   GDYGEVTLDCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALPWTSPSSTAWRNRELLMEFEEAHATKQSVVALGSQEGGLHQALAGAI
NAKAYAMA    ----------------------------------------------P-------------------------------------------
BEIJING-1   ------------------------------------------------------------------------------------------
KAMIYAMA    ----------------K-------------------------------------------------------------------------
69004       ----------------------------------------------P-------------------------------------------
MUAR        -------M-------F-------L---T----------N------L-----N-----I-L--------------------A---------
44/92       -----------------------------------------S------------------------------------------------
67/93       -----------------------------------------S------------------------------------------------
JaOH0566    ------------------------------------------------------------------------------------------

                                                                                                     360
JaOArS982   VVEYSSSVKLTSGHLKCRLKMDKLALKGTTYGMCTEKFSFAKNPADTGHGTVVIELSYSGSDGPCKIPIVSVASLNDMTPVGRLVTVNPF
NAKAYAMA    ------------------------------------------------------------------------------------------
BEIJING-1   ------------------------------------------------------------------------------------------
KAMIYAMA    ------------------------------------------------------------------------------------------
69004       --------------------------------------------------------------A---------------------------
MUAR        ----------------------------------------S---------------Q-T-T--------S-------L------------
44/92       ----------------------Q---------------------------------T-----------------------G---------
67/93       --------------------------------------------------------T------S--------------------------
JaOH0566    ------------------------------------------------------------------------------------------

                                                                                                     450
JaOArS982   VATSSANSKVLVEMEPPFGDSYIVVGRGDKQINHHWHKAGSTLGKAFSTTLKGAQRLAALGDTAWDFGSIGGVFNSIGKAVHQVFGGAFR
NAKAYAMA    ---------------------------------Q--------------------------------------------------------
BEIJING-1   ------------------------------------Y-----------------------------------------------------
KAMIYAMA    ------------------------------------------------------------------------------------------
69004       ---------------------N----------------V---------------------------------------------------
MUAR        ----T--------L-------F-------------L--V-------------------V----D-----T------------H----T--
44/92       -----S------G-----------------------------------------------------------------------------
67/93       -----S------------------------------------------------------------------------------------
JaOH0566    ------------------------------------------------------------------------------------------

                                                         500
JaOArS982   TLFGGMSWITQGLMGALLLWMGVNARDRSIALAFLATGGVLVFLATNVHA
NAKAYAMA    --------------------------------------------------
BEIJING-1   ----------------------I---------------------------
KAMIYAMA    --------------------------------------------------
69004       --------------------------------------------------
MUAR        -----------------------K---G----G-----------------
44/92       -----------------------------------------A--------
67/93       --------------------------------------------------
JaOH0566    --------------------------------------------------
```

Fig. 5. Alignment of the amino acid sequences of the E-proteins of 44/92 and 67/93 strains with the corresponding sequences of other 7 JE virus strains [12, 41]. Dots indicate that amino acid is the same as in JaOArS982. Sequences are shown in the single-letter amino acid code. Amino acids are numbered from the first amino acid of the E-protein

to threonine, and 366, alanine to serine, compared with the JaOArS982 strain. Three of the four substitutions caused a change in polarity and/or hydrophilicity (Table 4). Amino acid sequences around positions 327 and 366 are hydrophilic and possess a β-turn structure. Two substitutions at positions 222 and 366 could interfere with the formation of α-helices.

Discussion

Serological differences among JE virus strains was first described by Hale and Lee in 1954 [11]. Using antigen-adsorbed antisera in the HI and complement-fixation tests, Okuno et al. in 1968 showed that 26 JE virus isolates were classified into Nakayama and JaGAr01 immunotypes [32]. Oya reported that multiple JE virus strains can be classified either into Nakayama or Beijing-1 (which includes JaGAr01) subtype in the cross-N tests using immune mouse sera raised against each strain JE vaccine [34]. Through monoclonal antibodies, Kimura-Kuroda and Yasui demonstrated that JE virus envelope glycoprotein (E) carries multiple epitopes with biological activities including hemagglutination and neutralization [21]. Based on the reactivities of monoclonal antibodies, Kobayashi et al. [23, 24] have reported four antigenically distinct groups by examining JE virus strains from Japan and Southeast Asia. Subsequently, sequence analysis of the structural proteins of these antigenic groups has revealed variations at both nucleotide and amino acid levels, in agreement with the result of monoclonal antibodies [12]. On the basis of their findings, Hasegawa et al. [12] proposed that monoclonal antibodies may be useful to compare the antigenic differences among JE virus strains.

On the other hand, genomic heterogeneity among various JE virus isolates was demonstrated by the oligonucleotide fingerprint analysis [1,17,18]. These studies, in general, showed that the strains with geographically and chronologically closely related strains possessed higher similarities. More recently, by analyzing 240 nucleotide sequence in the PrM gene region, Chen et al. [4,5] classified a number of JE virus isolates from a variety of geographic areas in Asia into 3–4 genotypes.

Five amino acid changes specific to the 44/92 strain were present primarily in the R2 domain of the flavivirus E proteins, which is immunologically important [28]. One of the 5 substitutions, at position 373, is highly conserved among flaviviruses, such as JE, Murray Valley encephalitis (MVE), West Nile (WN), St. Louis encephalitis (SLE), yellow fever-17D (YF-17D), dengue and tick-borne encephalitis (TBE) viruses (Table 5). Whereas, another substitution at position 293 is conserved among JE, MVE, WN, SLE, YF-17D, and dengue type 1 flaviviruses [25]. Of these two conserved residues, the one at 293 exists in the cysteine-rich region. Four of the five 44/92-specific substitutions caused a change in polarity and/or charge. Substitutions at positions 293 and 373 introduced neutral residues which could interfere with the formation of α-helices and affect the secondary or tertiary structure of the E protein.

In the case of the 67/93 strain, the only substitution at residue 334 from proline to serine is strain-specific and unique among all flaviviruses (Table 5), and could change the polarity from hydrophobic to hydrophilic. The amino acid sequence around this residue is hydrophobic.

Table 5. Amino acid differences in the corresponding position of 44/92 and 67/93 with the other members of the flavivirus

Strains[a]	129	131	222	293	327	334	351	366	373	492
JE	T	Q	A	K	S	P	V	A	E	V
44/92	M	H	S	Q	T	.	G	S	G	A
67/93	M	.	S	.	T	S	.	S	.	.
MVE	L	L	L	.	Q	.	.	.	.	L
WN	I	.	N	.	Q	.	.	.	.	L
SLE	.	L	N	.	Q	.	.	.	.	I
YF-17D	E	D	T	.	K	.	K	D	.	M
DEN-2	V	L	P	R	Q	.	L	S	.	L
DEN-1	I	.	P	.	K	.	N	K	.	L
DEN-4	L	R	P	R	K	.	.	S	.	L
TBE	G	V	.	G	T	.	A	G	.	L

Numbers of the top line indicate the position of amino acid in the deduced amino acid sequence of E protein of JE virus strain JaOArS982 [41]. Numbers are counted from the N-terminal of E protein. Dots indicate amino acid is the samse as in JaOArS982. *JE* Japanese encephalitis, *MVE* Murray Valley encephalitis, *WN* West Nile, *SLE* St. Louis encephalitis, *YF* yellow fever, *DEN-1, 2, 4* dengue serotype 1, 2 and 4, *TBE* tick-borne encephalitis

[a] Ref. [25]

Four amino acid changes were identified in 44/92 and 67/93 that were different from all seven JE virus strains with published sequences. Two of the four changes at residues 222 and 366 could interfere with the formation of α-helices and affect the secondary or tertiary structure of the protein.

The significance of such amino acid alteration in relation to virulence or antigenicity is not yet clear. Some of the strain-specific changes, such as at position 293, 373 or 334, occurred in the highly conserved position among flaviviruses, suggesting that some biological alterations were caused by these substitutions. Since the E protein of flaviviruses determines virus tissue tropism, cell fusion or infection, virus maturation and induction of protective immunity, the observed changes in the amino acid sequences of these two strains might have some roles in the pathogenesis. Moreover, mutations on the E protein of flaviviruses which affect the pathogenicity have been well documented in several studies and proved that a change in amino acid could affect the virus to cell attachment or neutralization or virulence [3, 13, 15, 25]. Further experiments are needed to establish whether those residues are biologically important or not.

The data on the E protein gene sequence of 44/92 and 67/93 strains presented here are consistent with their unique serological reactions in the HI and N tests. It still remains to assign these strains into any of the antigenic groups, either by their reactivities with monoclonal antibodies or polyclonal antibodies raised against some JE virus strains other than Nakayama or JaGAr01 strains. In addition, the epidemiological significance of these two isolates remains to be established.

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