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OLIGONUCLEOTIDE FINGERPRINT ANALYSIS ON JAPANESE ENCEPHALITIS VIRUS STRAINS ISOLATED IN JAPAN AND THAILAND

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Summary. — Genetic variation in Japanese encephalitis (JE) virus isolates from Japan and Thailand was examined by oligonucleotide fingerprints of the 42S genome RNA. Japanese isolates were rather similar to each other, as were the recent isolates from Thailand. However, recent Thai isolates were significantly different from recent Japanese isolates. From these results it was presumed that mutations and selections of the JE virus genome would have progressed independently in these geographically distant areas.

Key words: *Japanese encephalitis virus*; *flavivirus*; *oligonucleotide fingerprint*; *geographical distribution*

Introduction

JE virus is a member of Flaviviridae with single-stranded RNA genome of 42S. Since the first isolation of this virus in 1935, a lot of strains have been isolated from mosquitoes, swine, and horses, as well as from fatal human brains in Japan. The virus is known to exist not only in Japan but also in Korea, China, Taiwan, and Southeast to South Asia. Many researches on this virus have been carried out from epidemiological to molecular biological fields. Analysis of the viral RNA genome by fingerprinting of the RNase-T1-resistant oligonucleotides, as described by Wachter and Fiers (1972), has been proved to be useful in ecological and epidemiological studies to reveal strain differences of various viruses, for example, influenza virus (Nakajima *et al.*, 1980), poliovirus (Nottay *et al.*, 1981), St. Louis encephalitis virus (Trent *et al.*, 1981), dengue type 1 virus (Repik *et al.*, 1983), dengue type 2 virus (Trent *et al.*, 1983), yellow fever 17D vaccine (Monath *et al.*, 1983) and enterovirus 70 (Takeda *et al.*, 1984). We have analyzed the oligonucleotide fingerprints of 12 strains of JE virus isolated in Japan and Thailand in various years to acquire the knowledge of molecular epidemiology of the virus.

Materials and Methods

Cells. Aedes albopictus clone C6/36 cells (Igarashi, 1978) were grown at 28°C in Eagle's minimal essential medium (MEM, Eagle, 1959) containing heat-inactivated 10% fetal calf serum and 0.2 mmol/l each of 7 non-essential amino acids.

Table 1. JE virus strain used in comparative oligonucleotide fingerprint studies

Strain	Year	Place	Source
	Japanese isolations		
Nakayama RFVL	1935	Tokyo	Human CSF
JaOHO566	1966	Osaka	Human brain
Mie44-1	1969	Mie	Mosquito
B-18A(0)	1978	Osaka	Mosquito
JaOArS982	1982	Osaka	Mosquito
JaNAr9483	1983	Nagasaki	Mosquito
JaNAr13383	1983	Nagasaki	Mosquito
	Thai isolations		
Chiangmai	1964	Chiangmai	Human brain
P-19-Br	1982	Chiangmai	Human brain
KEO87	1983	Kampangphet	Human brain
ThCMAr4884	1984	Chiangmai	Mosquito
ThCMAr18084	1984	Chiangmai	Mosquito

CSF: cerebrospinal fluid

Virus. Table 1 lists the year of isolation, geographical location and the source of each JE virus strain examined in this study. Chiangmai was obtained from Dr. Oya, National Institute of Health of Japan, and KEO87 strain was received from Dr. C. Hoke, Armed Force Research Institute of Medical Science, Bangkok. JaOArS982 was received from Osaka Prefectural Institute of Public Health. Nakyama RFVL and Mie44-1 were received from Prof. Kobayashi, Department of the 1st Internal Medicine, Medical School of Ehime University. JaNAr9483, JaNAr13383, P-19-Br, ThCMAr4884 and ThCMAr18084 were isolated by our group. Every strain was grown one to 3 times in C6/36 cells to make seed stocks.

Preparation of the virus specimens. A mass culture of C6/36 cells was prepared in a spinner culture bottle at 28 °C with 500 ml of cell growth medium containing 2 mg/ml of microcarrier (Cytodex-1, Pharmacia, Sweden). After 48 hr of incubation, growth medium was removed, and the cells were infected with 20 ml of seed virus. The virus was allowed to adsorb for 2 hours at room temperature and 450 ml/bottle of maintenace medium (cell growth medium in which serum concentration was reduced to 2 %) was added. The culture was incubated for 48 hr at 28 °C and infectious fluid was collected. Cell debris and microcarriers were removed by centrifugation at 2500 rev/min for 15 min. To supernatant were added polyethylene glycol 6000 to concentration of 6 g/dl and NaCl to 0.5 mol/l, respectively, and the virus was precipitated for 15 minutes. After centrifugation (10,000 × g, 30 min), the supernatant was removed and the precipitate was resuspended in 8 ml of STE buffer (0.1 mol/l NaCl, 0.01 mol/l Tris-HCl, 1 mmol/l EDTA, pH 7.6). The suspension was centrifuged at 2,500 rev/min for 15 minutes. The supernatant was loaded over 4 ml of 15 % sucrose in STE buffer and centrifuged at 37,000 rev/min for 120 min in a SW-41 rotor of a Beckman L5-50 ultracentrifuge. The supernatant was removed and the pellet was used for RNA extraction.

Preparation of virion RNA. Partially purified and pelleted virions were solubilized with 0.1 % of sodium dodecyl sulfate (SDS) in STE buffer and RNA was immediately extracted twice with an equal volume of phenol saturated with STE buffer. The RNA was precipitated from the aqueous phase with 2 volumes of ethanol at −20 °C overnight. Then it was pelleted by centrifugation at 15,000 rev/min for 30 min, and vacuum dried. The RNA pellet was resuspended with 0.1 % SDS in 0.2 ml of STE buffer, and centrifuged in 15−30 % (w/w) linear sucrose gradient in 0.1 % SDS-STE buffer at 45,000 rev/min for 180 min at 20 °C in a SW-50.1 rotor. Fractions were collected by an ISCO gradient fractionator model 640, reading the OD at 254 nm. The peak fractions of 42S RNA were pooled and precipitated with ethanol and vacuum dried again.

Table 2. Similarity ratios among JE virus isolates as determined by RNA oligonucleotide fingerprint analysis*

Country	Strain	Year	Naka	0566	Mie	B-18	S982	9483	3383	Chia	P-19	KE	4884	8084
Japan	Nakayama RFVL	1935	—	.57	.64	.61	.69	.71	.67	.57	.52	.58	.52	.54
	JaOHO566	1966	.57	—	**.81****	.60	.58	.70	.69	.59	.51	.60	.51	.52
	Mie44-1	1969	.64	**.81**	—	.63	.68	**.83**	**.78**	.74	.57	.60	.58	.56
	B-18A(O)	1978	.61	.60	.63	—	.74	.63	.63	.63	.46	.44	.42	.41
	JaOArS982	1982	.69	.58	.68	.74	—	**.76**	**.75**	.55	.56	.54	.51	.56
	JaNAr9483	1983	.71	.70	**.83**	.63	**.76**	—	**.90**	.67	.60	.60	.58	.59
	JaNAr13383	1983	.67	.69	**.78**	.63	**.75**	**.90**	—	.63	.62	.63	.61	.62
Thailand	Chiangmai	1964	.57	.59	.74	.63	.55	.67	.63	—	.54	.53	.58	.56
	P-19-Br	1982	.52	.51	.57	.46	.56	.60	.62	.54	—	.71	**.81**	**.81**
	KEO87	1983	.58	.60	.60	.44	.54	.60	.63	.53	.71	—	**.77**	**.78**
	ThCMAr4884	1984	.52	.51	.58	.42	.51	.58	.61	.58	**.81**	**.77**	—	**.91**
	ThCMAr18084	1984	.54	.52	.56	.41	.56	.59	.62	.56	**.81**	**.78**	**.91**	—

* Naka; Nakayama RFVL, 0566; JaOHO566, Mie; Mie44-1, B-18; B-18A(O), S982; JaOArS982, 9483; JaNAr9483, 3383; JaNAr13383, Chia; Chiangmai, P-19; P-19-Br, KE; KE087, 4884; ThCMAr4884, 8084; ThCMAr18084.

** Ratios above 0.75 are bold type.

Oligonucleotide fingerprinting. The procedures of labeling and fingerprinting were essentially the same as described by Pedersen and Haseltine (1980). The purified RNA was digested with 2.5 units of RNase-T1 (Sankyo Pure Chemicals Co., Japan) at 37 °C for 60 min. The 5 -end of oligonucleotide was labeled with 370 kBq of γ — ^{32}P-ATP (5,000 Ci/mmol, Amersham International plc, England) with 2.5 units of polynucleotide kinase (Boehringer Mannheim, West Germany) at 37 °C for 20 hr. The reaction was stopped by addition of the ammonium acetate and yeast RNA mixture to 0.3 mol/l and 1 mg/ml final concentration respectively. The labeled oligonucleotides were precipitated from the mixture with 2 volumes of ethanol at −20 °C overnight, collected by centrifugation at 15,000 rev/min for 30 min and vacuum dried. Two dimensional polyacrylamide gel electrophoresis of ^{32}P-labeled oligonucleotides was carried out by the procedure described by Wachter and Fiers (1972) with some modifications. The first dimension of electrophoresis was carried out at 4 °C in 7.2 % (w/v) polyacrylamide gel containing 6 mol/l urea adjusted to pH 3.3 with citric acid until the dye marker (BPB) reached 15 cm from the starting point. The second dimension of electrophoresis was carried out at room temperature in 22 % (w/v) polyacrylamide gel in 50 mmol/l Tris-borate, pH 8.2, until the BPB reached 20 cm from the lower edge of the first gel piece. The gel was exposed to X-ray film (Sakura, Japan) with intensifying screen at 4 °C for appropriate time.

Calculation of the similarity ratio. The similarity ratio (SR) was calculated as described by Morita and Igarashi (1984) by the following formula: $SR = 2C/(A + B)$, where A is the number of large oligonucleotide spots in one strain, B is the number of large oligonucleotide spots in another strain, and C is the number of large oligonucleotide spots common to both strains.

Results

Fig. 1 shows the fingerprint patterns and their diagrams of 3 representative Japanese strains, and Fig. 2 shows those of 3 representative Thai strains. Unique large oligonucleotide spots in each fingerprint were arbitrarily numbered as shown in the diagrams in order to compare them among examined strains. Fig. 3 shows the composition of these spots observed in 12 strains of JE. All Japanese isolates shared some large oligonucleotide spots in common (No. 9, 10, 14, 19, 23, 42, 69). Spot No. 69 was in Japanese strains, while spot No. 22 was unique in Thai strains. Several spots were observed only in a single or a few strains. JaNAr13383 possessed spot No. 35 and 36. Two Japanese strains isolated in 1983 shared spot No. 50. Only Nakayama RFVL possessed spot No. 58. JaOArS982 possessed spot No. 65 and 66. Mie44-1

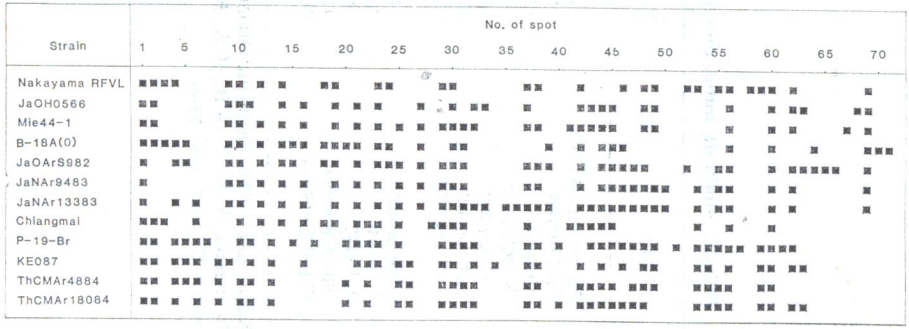

Fig. 3.
Comparison of large oligonucleotide spots in 12 strains of JE virus
The symbol (■) indicates the existence of a spot.

possessed spot No. 67. JaOHO566 possessed spot No. 68. B-18A(O) possessed spot No. 70 and 71. P-19-Br possessed 5 unique sposts (No. 7, 17, 51, 57, 61). Thai strains isolated in 1982, 1983 and 1984 shared spot No. 13. Chiangmai possessed spot No. 28, and KEO87 possessed spot No. 34.

Table 2 shows similarity ratios (SR) among 12 strains of JE shown in Fig. 3. All the Japanese isolates were relatively similar to each other (SR: more than 0.57) and the similarity was generally greater as the years of isolation were closer. For example, SR among the isolates in 1966 and 1969 were high (SR: 0.81) though Mie44-1 showed high SR (0.83—0.78) to JaNAr9483 and JaNAr13383, as were the strains in 1982 and 1983 (SR: more than 0.75). Especially the isolates in the same place in the same year showed high SR, as in the case of JaNAr9483 and JaNAr13383 (Nagasaki, Japan, 1983) which revealed SR = 0.90. Recent (1982—1984) isolates in Thailand were very similar (SR: 0.77—0.91) except one combination showing SR = 0.71. Especially SR between ThCMAr4884 and ThCMAr18084 (both isolated in Chiangmai, 1984) was very high (SR: 0.91). These recent Thai isolates showed generally low SR to recent Japanese strains isolated after 1982 (SR: 0.51—0.63).

Discussion

It was reported th at the isolates in the same geographical area and in the same year were very similar but differed from those in other areas or in different years in the same area, as shown by Trent *et al.* (1981) for St. Louis encephalitis virus, by Repik *et al.* (1983) for dengue type 1 virus, by Trent *et al.* (1983) for dengue type 2 virus and by Morita and Igarashi (1984) for Getah virus. The genome change with poliovirus during passage through humans was shown by Nottay *et al.* (1981), and with yellow fever 17D vaccine during egg passages by Monath *et al.* (1983). Takeda *et al.* (1984) documented the constellation of enterovirus type 70 isolates giving rise to a conical shape with axis of time lapse and circle of geographical area, when the isolates were arranged three-dimensionally according to the number of base changes. Our result also showed that recent JE virus strains in Japan were significantly different from those in Thailand in their genome RNA, although an old Thai strain (Chiangmai, 1964) appeared to have some degree of similarity to Japanese strains. The result suggests that mutations and selections of JE virus genome would have progressed independently in these two areas, which are sufficiently distant from each other. The reason why B-18A(O) was not very similar to the strains isolated in close years, although the SR to JaOArS 982 in 1982 was 0.74, is difficult to explain. B-18A(O) was obtained as the first isolate during epidemic period in 1978 in Osaka (Igarashi *et al.*, 1981). Some factors, which we have not found yet, might cause rapid changes in genome RNA for 4 years. Further analysis on other isolates in 1978 or in the following years in Osaka may be necessary in order to substantiate this reasoning. Also the similarity between strains in Japan and those in neighbouring areas like China and Korea would have to be examined in further analyses in order to acquire some clue to solve the question whether the virus persists

in Japan during the winter season or it will be introduced every summer from other countries.

Strain differences as revealed by antigenic variations have been reported on JE virus and West Nile virus (Hammam *et al.*, 1965, 1966, and Okuno *et al.*, 1968). Recently Hasegawa *et al.* (1982), Kobayashi *et al.* (1983), Kimura--Kuroda and Yasui (1983) reported that JE virus strains can be classified according to their reactions with monoclonal antibodies. This grouping was also observed by the antigenicities of several JE virus strains, examined by their capcity to produce neutralizing antibodies by respective vaccine strains (Takagi *et al.*, 1984). According to these grouping, JaOHO566 and Mie44-1 were similar to Nakayama. These results do not completely agree with our results of fingerprint analysis. Since antigenic variations are dependent on the fine structural change of antigenic sites, especially those on the major structural glycoprotein, GP58 (Trent, 1977), the result of antigenic analysis does not necessarily agree with the result of fingerprints, which reflects the change in the entire genome RNA.

We exposed the gel to X-ray film with intensifying screen at 4 °C. However, preflash X-ray film in combination with intensifying screen exposed at —70 °C should be used for further studies in order to detect weak spots.

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Explanation to Figures (Plates XL—XLI):

Fig. 1. The RNase-T1-resistant oligonucleotide fingerprint patterns and their diagrams of 3 representative Japanese strains. Unique large oligonucleotide spots in each fingerprint were arbitrarily numbered. (A) Nakayama RFVL (1935), (B) Mie44-1 (1969), (C) JaNAr13383 (1983).

Fig. 2. The oligonucleotide fingerprint patterns and their diagrams of 3 representative Thai strains. (A) Chiangmai (1964), (B) KEO87 (1983), (C) ThCMAr18084 (1984).