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RNA Oligonucleotide Fingerprint Analysis of Dengue Serotype 3 and 4 Viruses Isolated in the Southeast Asia

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Abstract: Oligonucleotide fingerprints of the 42S genome RNA of dengue (DEN) serotype 3 and 4 virus isolated in the Southeast Asia were examined. DEN type 3 (DEN−3) virus strains were apparently different from DEN type 4 (DEN−4) virus strains in their fingerprint patterns. Two DEN−3 isolates in the Philippines were rather similar each other, and also three DEN−3 isolates in Indonesia were rather similar, whereas the isolates in the Philippines, Indonesia, and Thailand were significantly different from each other. The result suggests that mutations and selections of DEN−3 viral genome would have progressed independently in these geographically distant areas. The DEN−4 virus isolated from the serum of a patient with laboratory infection and passaged 10 times in suckling mouse brain (SMB) was considerably similar to its parent strain, suggesting that the viral genome would not have changed so much by these passages.

Key words: Dengue serotype 3 and 4 viruses, Oligonucleotide fingerprint, Geographical distance, Passage history

INTRODUCTION

DEN virus types 1, 2, 3, and 4 are distinguishable serologically, and have been paid so much attention during the past 30 years as causative agents of dengue hemorrhagic fever (DHF) and dengue fever (DF) in the Southeast Asia. Analysis of the viral RNA by fingerprinting of the RNase−T1−resistant oligonucleotide, as described by Wachter and Fiers (1972), has been used to compare the strain differences and similarities of various viruses. Such molecular epidemiological studies have been reported for influenza virus (Nakajima *et al.*, 1980), poliovirus (Nottay *et al.*, 1981), St. Louis encephalitis virus (Trent *et al.*, 1981), yellow fever 17D vaccine (Monath *et al.*, 1983),

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enterovirus 70 (Takeda *et al.*, 1984), Getah virus (Morita and Igarashi, 1984), and Japanese encephalitis virus (Hori *et al.*, 1986). In the case of DEN viruses, differences among 4 prototype viruses have been reported by Vezza *et al.* (1980), followed by strain differences of type 1 (Repik *et al.*, 1983) and type 2 (Trent *et al.*, 1983) viruses. We have analyzed the oligonucleotide fingerprints of DEN 3 and 4 virus strains to acquire the knowledge of molecular epidemiology of these viruses in the Southeast Asia.

Materials and Methods

Virus strains: Table 1 lists DEN 3 and 4 virus strains used in this study. DEN−3; H−87 is the prototype strain. The strain of 16562 (Halstead *et al.*, 1970 a) was received from Virus Research Institute, Thailand, by permission of Dr. Halstead. V−11 isolated by Dr. Rosen, and was obtained from Dr. Inoue, National Institute of Health of Japan. D80−753 was received from Virus Research Institute, Thailand. U20−82 and I6−82 were isolated by our group (Igarashi *et al.*, 1982). DEN−4; 4328−S (Halstead *et al.*, 1970 b) was received from Virus Research Institute, Thailand, by permission of Dr. Halstead. No. 17 was isolated by Dr. Rosen and obtained from Dr. Inoue. No. 124 was isolated in Thailand (Fukunaga *et al.*, 1980), SI−YO was isolated from patient's serum of laboratory infection by No. 124 and passaged 10 times in SMB (Fukunaga *et al.*, 1982). These 2 strains were obtained from Research Institute for Microbial Diseases of Osaka University.

Preparation of the virus specimens and virion RNA: The procedure was as described elsewhere (Hori *et al.*, 1986). A mass culture of *Aedes albopictus* clone C6/36 cells (Igarashi, 1978) was infected with seed virus and the infected culture fluid was

Table 1. Dengue virus strains used in comparative oligonucleotide fingerprint studies

Serotype	Strain	Country	Year	Disease*
Dengue 3	H−87	Philippines	1956	DHF
	16562	Philippines	1964	DHF
	V−11	Indonesia	1976	DF
	I6−82	Indonesia	1982	DHF
	U20−82	Indonesia	1982	DF
	D80−753	Thailand	1980	?
Dengue 4	4328−S	Philippines	1966	DHF
	No. 17	Sri Lanka	1978	?
	No. 124	Thailand	1978	DHF
	SI−YO	Thailand	1978	DF

*DHF, Dengue hemorrhagic fever, DF, Dengue fever.

harvested after 5 to 7 days. After low speed centrifugation, virion was concentrated and partially purified from the supernatant by polyethylene glycol precipitation and ultracentrifugauion. RNA was extracted from the pellet with phenol, ethanol precipitated and was purified by sucrose gradient sedimentation. The peak fractions of 42S RNA were collected, ethanol precipitated and used as purified virion RNA.

Oligonucleotide fingerprinting: The procedure of oligonucleotide fingerprinting was essentially the same as described by Pedersen and Haseltine (1980). The 5'−end of oligonucleotides obtained by digesting virion RNA with RNase−T1, were labeled by $\gamma-^{32}P-ATP$ with polynucleotide kinase (Boehringer Mannheim. West Germany). Two dimensional polyacrylamide gel electrophoresis of ^{32}P−labeled oligonucleotides was carried out by the procedure described by Wachter and Fiers (1972), using 7.2% gel at pH 3.3 for the first dimensional, and 22% gel at pH 8.0 for the second dimensional electrophoresis, respectively. The gel was exposed to X−ray film to prepare fingerprints.

Calculation of 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 unique large oligonucleotide spots in one strain, B is the number of unique large oligonucleotide spots in another strain, and C is the number of unique large oligonucleotide spots common to both strains.

RESULTS

Fig. 1 shows the fingerprint patterns and their diagrams of 2 representative DEN−3 strains, and Fig. 2 shows those of 2 DEN−4 strains. Fingerprint patterns of DEN−3 virus strains were apparently different from those of DEN−4 virus strains, therefore we investigated DEN−3 virus and DEN−4 virus separately. 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 6 strains of DEN−3 virus, and Fig. 4 shows similar results for 4 strains of DEN−4 virus.

Table 2 shows similarity ratios (SR) among 6 strains of DEN−3 virus. SR between H−87 and 16562 in the Philippines was very high (SR: 0.93), although there was approximately 10 years difference between the years of isolation. SR among isolates in Indonesia were also rather high (SR: 0.84−0.87). However, SR between the isolates in the Philippines and those in Indonesia were not very high (SR: 0.67−0.72). A Thai isolate did not show so high SR (0.57−0.67) to the Philippines and Indonesian isolates.

Table 3 shows SR among 4 strains of DEN−4 virus. Comparison of Sri Lanka and Thai isolates gave rather high similarity (SR: 0.77−0.80), however, SR between these isolates and the Philippines isolate were not high (0.60−0.64). The DEN−4 virus SI−YO isolated from the serum of a patient with laboratory infection and passaged 10 times in SMB showed considerably high SR(0.89) to its parent strain No. 124.

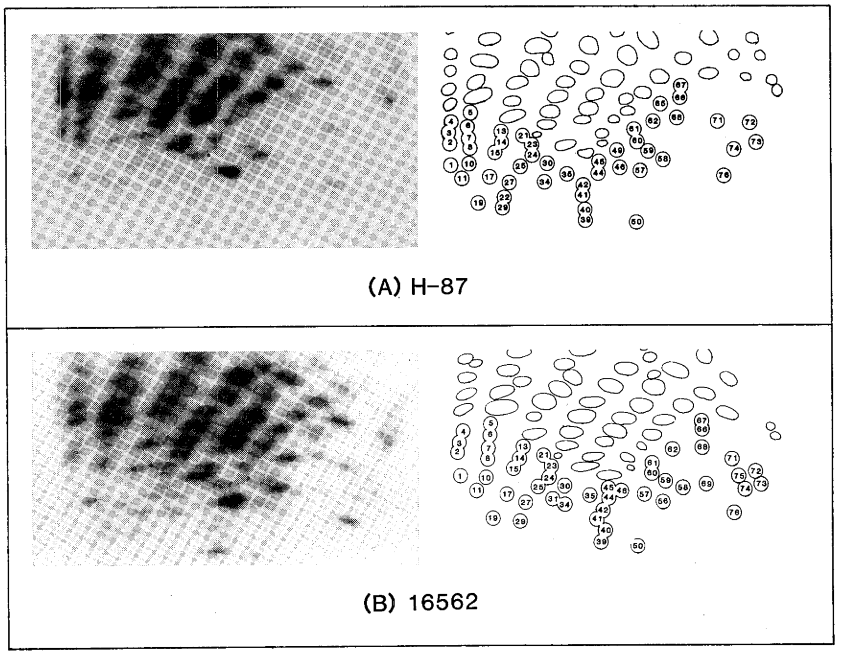

Fig. 1. The RNase−T1−resistant oligonucleotide fingerprint patterns and their diagrams of 2 representative dengue 3 strains. Unique large oligonucleotide spots in each fingerprint were arbitrarily numbered. (A) H−87 (Philippines, 1956), (B) 16562 (Philippines, 1964).

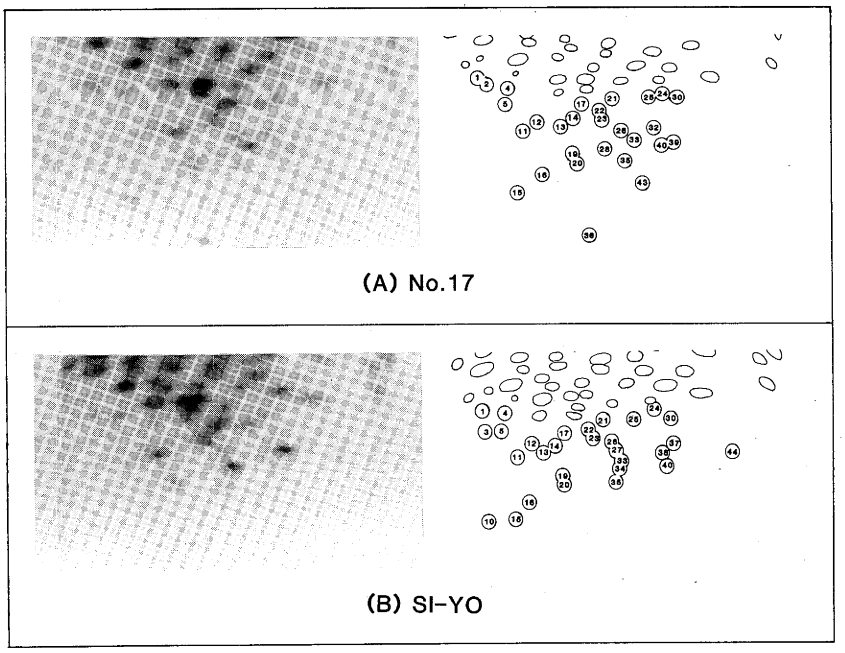

Fig. 2. The oligonucleotide fingerprint patterns and their diagrams of 2 representative dengue 4 strains. (A) No.17 (Sri Lanka, 1978), (B) SI−YO (Thailand, 1978).

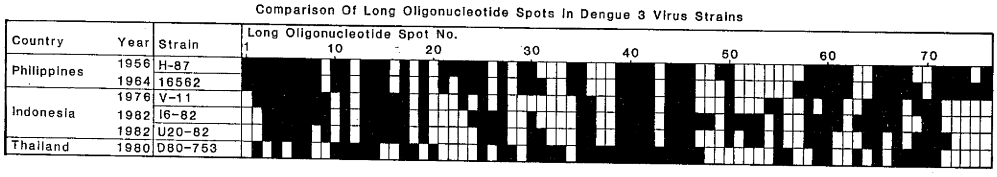

Fig. 3. Comparison of large oligonucleotide spots in 6 strains of dengue 3 virus. The symbol (■) indicates the existence of a spot.

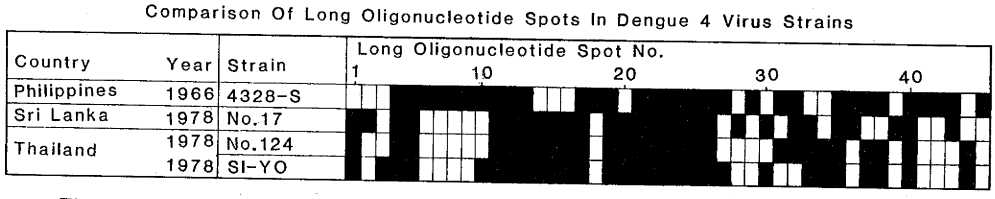

Fig. 4. Comparison of large oligonucleotide spots in 4 strains of dengue 4 virus. The symbol (■) indicates the existence of a spot.

Table 2. Similarity ratios among dengue 3 virus stratins as determined by RNA oligonucleotide fingerprint analysis*

Country	Year	Strain	H−87	1656	V−11	I682	U20	D80
Philippines	1956	H−87	−−	.93**	.72	.69	.67	.57
	1964	16562	.93	−−	.71	.67	.68	.60
Indonesia	1976	V−11	.72	.71	−−	.84	.84	.63
	1982	I6−82	.69	.67	.84	−−	.87	.63
	1982	U20−82	.67	.68	.84	.87	−−	.67
Thailand	1980	D80−753	.57	.60	.63	.63	.67	−−

*See Table 1.
**Ratios above 0.80 are underlined.

Table 3. Similarity ratios among dengue 4 virus stratins as determined by RNA oligonucleotide fingerprint analysis*

Country	Year	Strain	4328	No17	124	SIYO
Philippines	1966	4328−S	−−	.60	.64	.62
Sri Lanka	1978	No. 17	.60	−−	.80**	.77
Thailand	1978	No. 124	.64	.80	−−	.89
	1978	SI−YO	.62	.77	.89	−−

*See Table 1.
**Ratios above 0.80 are underlined.

DISCUSSION

It was described previously (Hori *et al.*, 1986) that analysis of the viral RNA genome by fingerprinting of the RNase−T1−resistant oligonucleotides had been proved useful in ecological and epidemiological studies. For example, Vezza *et al.* (1980) documented that in general, viruses separated serologically could easily be distinguished by oligonucleotide fingerprint, and in addition many isolates of identical serotype virus could be distinguished by this procedure. It was reported that 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 DEN type 1 virus, by Trent *et al* (1983) for DEN type 2 virus, by Morita and Igarashi (1984) for Getah virus, and by Hori *et al.* (1986) for Japanese encephalitis virus. DEN−3 virus strains were apparently different from DEN−4 virus strains in their RNA oligonucleotide fingerprints as Vezza *et al.* (1980) had reported, therefore, these two serotype DEN viruses were treated as different groups in our analysis. The present study revealed that the DEN−3 isolates in the same country were rather similar each other, whereas those in distant country were significantly different from each other. We had obtained similar result for Japanese encephalitis virus (Hori *et al.*, 1986). These results suggest that mutations and selections of DEN−3 viral genome would have progressed independently in distant area as Japanese encephalitis virus. The DEN−4 virus isolated from the serum of a patient with laboratory infection and passaged in SMB showed high SR to its parent strain. This fact suggests that DEN−4 viral genome would not have changed so much by these passage history. An isolate in Sri Lanka showed comparatively high SR to a Thai isolate in the same year. Though these 2 countries are rather distant from each other, the same or similar DEN−4 virus might have prevailed throughout these areas in 1980. Further analysis on more isolates in those days in these areas may be necessary in order to obtain more clear answer. Recently genetic relationships of DEN virus serotypes have been analyzed by cDNA−RNA hybridization experiments (Blok *et al.*, 1984, 1985). They estimated that sequence homology among four DEN serotypes showed variety (29−73%), and there was an average sequence homology of 54% between DEN−3 and DEN−4. Since unique large oligonucleotide spots in the fingerprint used to calculate SR in our study represent only a minor part of entire genome RNA, sequence homology among strains of the same serotype virus could be higher than by the SR, although fingerprint could give an answer of strain difference of similarities rather easily. No. 24 spot in Fig. 3 was shared only by the DEN−3 virus isolates from patients of DHF. Although we could not detect such spot for the DEN−4 virus isolates, we would think that etiology of DHF might also be related to the strain difference of the infecting virus, as well as to the immune response of the patients. These problems should require further studies on the molecular structure of viral genome including nucleotide sequencing.

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オリゴヌクレオチドフィンガープリント法によるデングウイルス3型と4型の解析

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東南アジアにて分離されたデングウイルス3型と4型を，RNase−T1 オリゴヌクレオチドフィンガープリント法により比較検討した．3型と4型の株間では，スポットのパターンは明らかに異なっていた．分離年代が10年ほど差があるフィリピンで分離された3型株は互いによく似ており，インドネシアで分離された3型株も互いによく似ていたが，フィリピン，インドネシア及びタイでそれぞれ分離された株では明らかに地域差がみられた．これらのことは，デングウイルス3型遺伝子の変異と撰択とが，これら地理的に充分離れた地域では，それぞれ独立して進行したことを示唆している．実験室内感染患者より分離後，乳のみマウス脳10代継代された4型ウイルスは患者に感染前の株とかなり類似していたことより，デングウイルス4型遺伝子はこの程度の継代ではあまり変化しないものと考えられる．

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