Application of Reverse Transcriptase−Polymerase Chain Reaction for Serotype Determination of Dengue Virus Strains Isolated in North Vietnam, 1991 to 1993

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Abstract: Reverse transcriptase-polymerase chain reaction (RT−PCR) was applied to determine serotypes of 20 dengue virus strains isolated in north Vietnam in the years of 1991 and 1993. There were 10 strains of dengue type 1 (D1), 9 strains of dengue type 2 (D2) and a single strain of dengue type 4 (D4), respectively. The results completely agreed with the serotypes determined by the immunofluorescent staining (IFA) of the infected cells using dengue type−specific monoclonal antibodies.

Key words: dengue virus, serotype, PCR

INTRODUCTION

Dengue virus infection has been a serious health problem in the tropical a[illegible] the world, with its growing magnitude in the number of cases and epidemic areas (Halstead, 1980; 1992). To make dengue situation worse is the appearance of severe clinical manifestation of dengue haemorrhagic fever (DHF), which has been epidemic in southeast Asia since 1953 and has been introduced into New World since 1981.

Laboratory diagnosis on dengue virus infection is important in order to provide accurate information on the disease agents. These informations are required for the clinicians, epidemiologists and health administrators for their proper case management, understanding real situation of the epidemic, and planning effective preventive measures. Due to the cross-reactivity among 4 dengue serotypes and among multiple flaviviruses, infecting virus type can be determined only by virus isolation. Classical serotyping which required cumbersome serological tests (Shope and Sather, 1979) was greatly simplified by introduction of serotype specific monoclonal antibodies in the IFA (Henchal *et al.*, 1982; 1983). However, Maneekarn *et al.* (1993) reported that some strains of D1 virus apparently did not possess reactivity to a

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D1−specific monoclonal antibody. In recent years, detection of dengue virus genome sequence and determination of their serotypes by using reverse transriptase polymerase chain reaction (RT−PCR) has been reported from several investigators (Deubel *et al.*, 1990; Morita *et al.*, 1991; Eldadah *et al.*, 1991; Henchal *et al.*, 1991; Lanciotti *et al.*, 1992; Chungue *et al.*, 1993). These procedures, although delicate and expensive, were reported to be much quicker and simpler than the conventional serotyping using monoclonal antibodies in the IFA of infected cells.

In this report, serotypes of 20 dengue virus stains isolated in north Vietnam were determined by the RT−PCR and compared with those by the IFA using monoclonal antibodies.

Materials and Methods

Dengue virus strains and serotype determination by IFA: Twenty strains of dengue viruses used in this study were isolated by the second author (T. N. Ninh) in Dengue Virus Laboratory, Department of Virology, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam. Serum specimens from dengue fever (DF) or dengue haemorrhagic fever (DHF) in north Vietnam in 1991 and 1993 were inoculated into *Aedes albopictus* clone C6/36 cells

Table 1. Dengue virus strains isolated in north Vietnam, 1991 and 1993, and their serotypes determined by the IFA and RT−PCR

Code of virus strains and years	Patient's record		Serotype of dengue virus determined by	
	Age and sex	Clinical diagnosis	IFA	RT−PCR
VN 222−91	36 M	DF	D2	D2
VN 227−91	29 M	DF	D1	D1
VN 228−91	30 M	DF	D1	D1
VN 229−91	6 F	DF	D1	D1
VN 235−91	39 M	DHF	D2	D2
VN 243−91	16 F	DHF	D2	D2
VN 244−91	13 M	DHF	D2	D2
VN 273−91	16 M	DHF	D2	D2
VN 283−91	19 M	DHF	D2	D2
VN 305−91	25 M	DHF	D2	D2
VN 317−91	33 M	DHF	D4	D4
VN 319−91	35 M	DHF	D2	D2
VN 320−91	15 F	DF	D4	D4
VN 367−93	15 F	DF	D1	D1
VN 371−93	36 F	DF	D1	D1
VN 373−93	5 M	DF	D1	D1
VN 374−93	18 F	DF	D1	D1
VN 375−93	33 F	DF	D1	D1
VN 376−93	11 M	DF	D1	D1
VN 378−93	32 F	DF	D1	D1

(Igarashi, 1978). The patients' data and clinical diagnosis were shown in Table 1. The infected cell smears were screened by the direct IFA using fluorescent dye labeled IgG prepared from high−titered DHF patients' serum. The positive specimens were then determined for their serotypes using type−specific dengue monoclonal antibodies in the indirect IFA (Henchal *et al.*, 1982; 1983; WHO, 1986).

Serotyping by RT−PCR: Each of the 20 strains of dengue viruses was inoculated into C6/36 cells and the infected culture fluid was collected 7 days after incubation at 28℃. The infected culture fluid was solubilized with detergent and subjected to the rapid and direct RT−PCR as described by Morita *et al.* (1991). Briefly, 5 μl of the infected C6/36 cell culture fluid was mixed with an equal volume of detergent mixture (1% Nonidet P40, 10 U of RNase inhibitor (Takara, Japan), in phosphate buffered saline) for 1 minutes at room temperature. The mixture was then added with 90 μl of RT−PCR mixture (100pmole each primer, 0.2 mM each deoxynucleoside triphosphates, 10 mM Tris−HCl, pH 8.9, 1.5 mM $MgCl_2$, 80 mM KCl, 0.5 mg/ml of bovine serum albumin, 0.1% sodium cholate, 0.1% Triton X−100, 10 U of reverse transcriptase (Life Science), and 2 U of Tth DNA polymerase (Toyobo, Japan). The reaction mixture was covered by 2 drops of mineral oil and the RT reaction was proceeded at 53℃ for 10 minutes. Then PCR amplification was carried out for 25 to 30 cycles which comprised of denaturation at 92℃ for 60 sec, annealing at 53℃ for 60 sec, and primer extension at 72℃ for 60 sec, in a thermal cycler (Iwaki, Japan). The last chain elongation step was extended to 10 minutes. Five microliters of the PCR reaction products were then subjected to agarose gel electrophoresis and the PCR products were visualized by ethidium bromide staining.

Results

Table 1 shows the results of serotypes determined on 20 test specimens by IFA and RT−PCR, together with the record of patients. Serotyping by RT−PCR completely agreed with the results by the IFA. There were 10 strains of D1, 9 strains of D2, and a single strain of D4, but type 3 was not detected. All 10 strains of D1 were isolated from DF cases (3 in 1991 and 7 in 1993, respectively). While 8 of the 9 strains of D2 were from DHF cases in 1991, and another D2 strain was from a DF case in 1991. A single D4 strain was isolated from a DF case in 1991.

Discussion

Previous reports from our laboratory demonstrated the potentiality of direct and rapid RT−PCR for serotype determination of dengue virus isolates in C6/36 cell culture fluid (Morita *et al.*, 1991; Maneekarn *et al.*, 1993). In these studies, serotypes of several dengue virus strains, which were isolated in south and southeast Asia such as Thailand, Philippines, Indonesia, and Sri Lanka, were successfully determined by the rapid RT−PCR using infected C6/36 cell culture fluid. Moreover, some of the D1 strains, which apparently lacked an

epitope of the D1−specific monoclonal antibody 15F3, was still determined as D1 by the RT−PCR. The method was then successfully applied to determine infecting serotypes of dengue virus in the patient's sera without prior virus isolation in C6/36 cells or RNA extraction. The examples were shown for imported dengue cases in Japan or under field setting in Northeast Thailand (Morita *et al.*, 1994; Igarashi, 1994). Present data provide another evidence to support the applicability of RT−PCR as the rapid and simple method to determine serotypes of dengue viruses including those circulating in north Vietnam in recent years.

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