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Principle of PCR and Its Application for the Diagnosis of Dengue and Japanese Encephalitis

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1. INTRODUCTION

The development of polymerase chain reaction (PCR) (1) has facilitated the diagnosis of a number of viral infections. It possesses several obvious advantages, such as high specificity, sensitivity, rapidity, etc. Now, PCR is a general diagnostic tool which is being applied to many viral infections.

On dengue and Japanese encephalitis (JE) virus, several groups of flavivirus investigators already applied this technique for rapid diagnosis (2, 3, 4, 5, 6), and all of them reported a potential of the technique for speedy detection and identification of dengue and JE virus.

Among those PCR procedures demonstrated so far, direct RT-PCR procedure (3) is the most speedy procedure in which sample specimens are subjected to PCR reaction without any RNA purification steps. The direct RT-PCR enables 2 to 3 hour diagnosis. In this paper, the principle and procedure of direct RT-PCR are explained, and the advantages and disadvantages of PCR diagnosis compared with serological examination, IgM-capture ELISA and IgG-capture ELISA, will be discussed according to the author's most recent data.

2. PRINCIPLE AND PRACTICAL PROCEDURE OF DIRECT RT-PCR FOR FLAVIVIRUS

a. Reverse transcription (RT):

Flavivirus possesses a single-stranded positive sense RNA genome. Therefore, reverse transcription by using reverse transcriptase (RTase) is an inevitable step for preparation of cDNA prior to PCR amplification (Fig. 1). Generally, viral RNA is extracted and purified by conventional RNA purification procedure, such as phenol-chroloform extraction and ethanol precipitation, and subjected to RT reaction. However, the characteristics of flavivirus virion structure enables direct RT reaction without RNA purification procedure. The flavivirus particle consists of a lipid bilayer with envelope (E) and membrane (M) proteins surrounding a spherical nucleocapsid composed of a genome RNA and capsid (C) proteins (Fig. 2). The virion structure is easily destroyed by treatment with non-ionic detergent or low osmoral shock. For example 1% Nonidet P-40 (NP-40) treatment can successfully disrupts dengue virus particles and does not, however, affect RT-PCR enzymic activity.

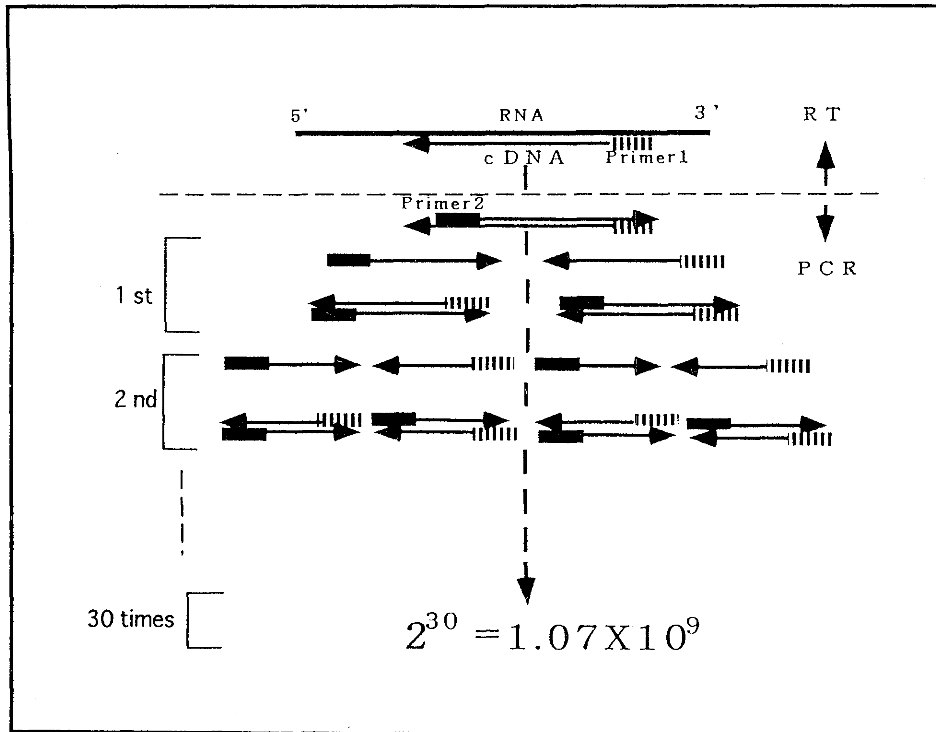

Fig. 1 Schema of RT-PCR

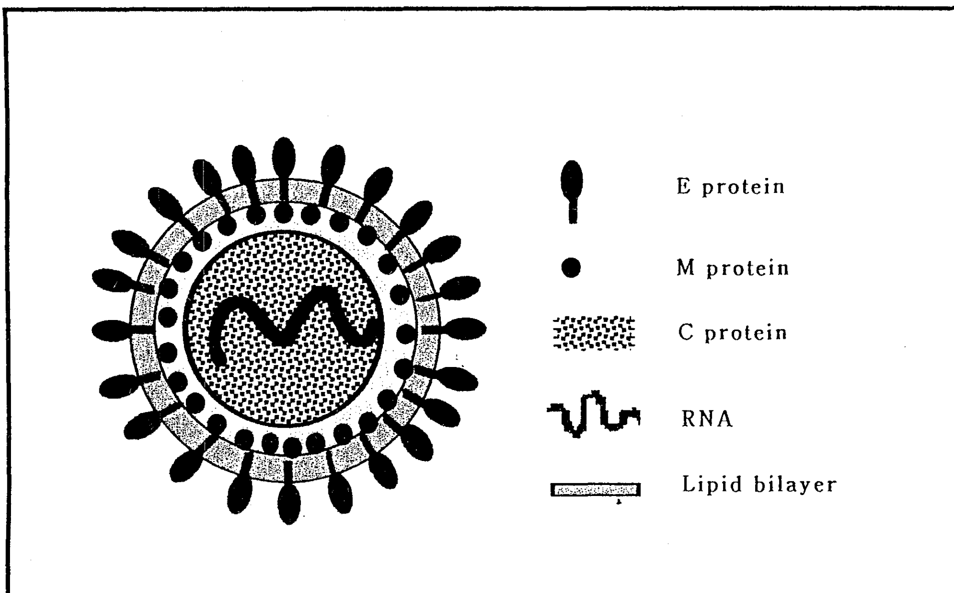

Fig. 2 Virion structure of flavivirus

b. Polymerase chain reaction (PCR):

One cycle of PCR is composed of three steps, that is, *denaturing* at 93℃, *annealing* of primers at 53℃ and *dsDNA synthesis* by heat stable DNA polymerase at 72℃. One cycle of PCR can double the amount of target NDA (Fig. 1), so, one copy of target DNA will be amplified to about 10^9 copies after 30 cycles amplification theoretically and the products can be detected on agarose gel electrophoresis.

c. Practical direct RT−PCR procedure:

RT and PCR procedure can be done sequentially in a single tube, because the buffer conditions for RT and PCR are almost identical. In short, five microliters of specimen is mixed with an equal volume of 1% Nonidet P-40 in PBS(−) with 100 units of RNasein (human placental RNase inhibitor) followed by 1 min. incubation at room temperature. Ninety microliters of RT-PCR mix (100 pmol of sense and reverse primers, 0.2mM dNTP, 10mM Tris (pH8.9), 1.5mM $MgCl_2$, 80mM KCl, 0.5mg/ml BSA, 0.1% sodium cholate, 0.1% Triton X−100, 10 units reverse transcriptase and 4 units Tth DNA polymerase, a thermostable DNA polymerase) were added to the above sample. The tubes were set in a thermal programmer, oil bath type PCR machine, and subjected to programmed incubation: 5 min. at 53℃ for reverse transcription once, followed by 30−40 PCR cycles (92℃ for 1 min., 53℃ for 1 min., 72℃ for 1 min. each). The entire procedure is shown as a schema in Fig. 3. After PCR amplification, 5 microliters of PCR product was mixed with 1 microliter of dye solution (1% SDS, saturated bromophenol blue and xylene cyanol) and subjected to agarose gel electrophoresis. Amplified DNA fragments were visualized by ethidium bromide staining.

3. Dengue PCR.

Primer sets for dengue PCR, which have been published by several groups (2, 3, 4, 5, 6), are listed in Table 1. Fig 4 is a photograph of an agarose gel stained with ethidium bromide containing RT-PCR products which were amplified from virus infected fluids by direct RT-PCR procedure using the primers, selected by Morita et al. (3). In this system, the target size of each dengue serotype (DEN 1, 2, 3, 4) has each unique length, enabling simultaneous identification and serotyping.

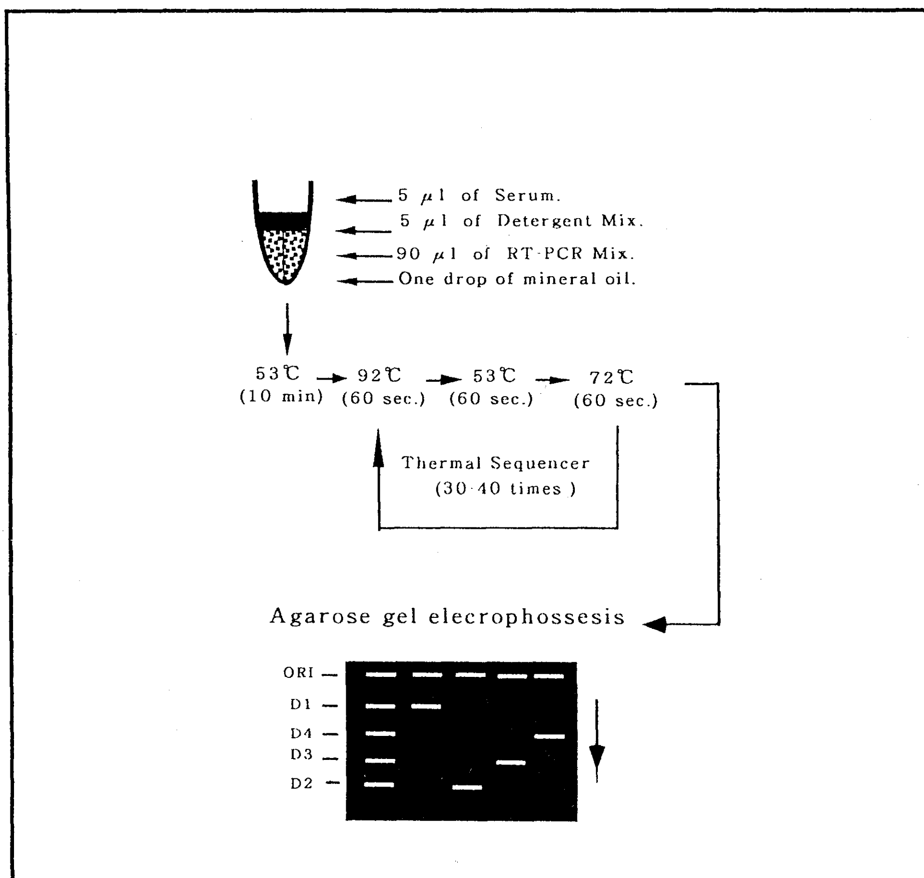

Fig. 3 Schema of direct RT-PCR procedure

Fig. 5 demonstrates the resultls of direct RT-PCR on imported dengue cases among three Japanese (7). Dengue virus type 1 genome was successfully amplified from all three cases at acute phase of the infection before sero-conversion. We examined 11 imported dengue cases so far and all of them (100%) showed PCR positive at acute phase. Sequential sera were obtained from one of the patients in Fig. 5 and examined by PCR, ELISA and virus isolation. PCR detectablel viremia and virus isolation were positive up to the 5th day from the onset of the disease in this case. Subsequent study, which was held in a dengue epidemic area with the NIH of Thailand and Nakhornphanom Privincial Hospital, clearly demonstrated that the PCR positive rate was high only at acute phase while ELISA (8) was negative and PCR detectable viremia disappeared quickly after the appearance of specific antibody (Vongcheree *et al.* manuscript in preparation). The results also demonstrated that more than 80% diagnostic efficiency was obtained at any phase of dengue infection when both PCR and ELISA examination were used.

Table 1. Nucleotide sequences of the primers for Dengue and JE.

		Size
1990. Deubel et al., J. Virol. Methods.		
D1 (+)	GCAACGTGGGTTGACGTGGTATTGG	237
D1 (−)	AAACGTTCGTCTACACACAAAGTTCG	
D2 (+)	GGGGTTTCAGGAGGAAGCTGGGTTGAC	266
D2 (−)	CCCCATCCTCTGTCTACCATG	
D3 (+)	CGGGAGCTACGTGGGTTGACGTGG	257
D3 (−)	CCAGCCTCTGTCTACGTATGTATGC	
D4 (+)	GGAGTCTCAGGTGGAGCATGGGTCGAC	267
D4 (−)	GCCCCACCCTCTGTCTACCACATC	
1991. Morita et al., J. Clin. Microbiol.		
D1S	GGACTGCGTATGGAGTTTTG	490
D1C	ATGGGTTGTGGCCTAATCAT	
D2S	GTTCCTCTGCAAACACTCCA	230
D2C	GTGTTATTTTGATTTCCTTG	
D3S	GTGCTTACACAGCCCTATTT	320
D3C	TCCATTCTCCCAAGCGCCTG	
D4S	CCATTATGGCTGTGTTGTTT	398
D4C	CTTCATCCTGCTTCACTTCT	
1991. Henchal et al., Am. J. Trop. Med. Hyg.		
AD3 (−)	CTGATTTCCAT(AGCT)CC(AG)TA	419
AD4 (+)	GA(CT)ATGGG(AGCT)TA(CT)TGGATAGA	
1991. Eldadah et al., J. Med. Virol.		
DEN−2−1	CGCGCTGCCCAACACAAGGG	400
DEN−2−2	GCTTTGTCTTTCATTTGCAG	
DEN−4−1	TTGTGGTTGACAACGTGCAC	440
DEN−4−2	GTTGGTCGTGAACATGCCAA	
1992. Lanciotti et al., J. Clin. Microbiol.		
D1	TCAATATGCTGAAACGCGCGAGAAACCG	511
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	
TS1	CGTCTCAGTGATCCGGGGG	482
TS2	CGCCACAAGGGCCATGAACAG	119
TS3	TAACATCATCATGAGACAGAGC	290
TS4	CTCTGTTGTCTTAAACAAGAGA	392
1992. Morita et al., Nippon Rinsho		
JEN3S	AACTCAACCGCAAGTCCTAT	116
JEN3R	GACCCTGCTCGCACCGAAGT	

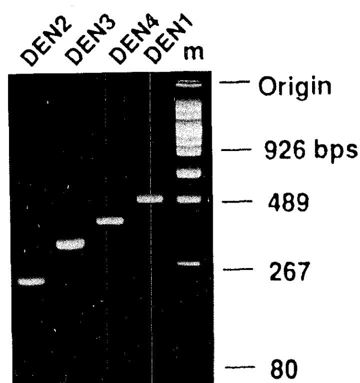

Fig. 4. Photograph of an agarose gel stained with ethidium bromide containing RT-PCR products. DEN 1−4: dengue type 1−4. m: DNA size marker.

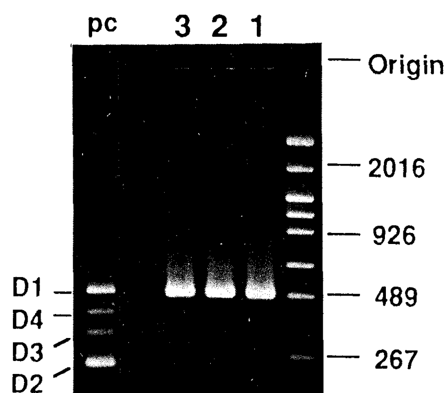

Fig. 5. Photograph of an agarose gel stained with ethidium bromide, containing RT-PCR products from three Japanese dengue patients. PC: Positive control, D1−4: dengue type 1−4. 1.2.3: Patient No. 1, 2 and 3.

4. JE PCR

We selected a primer pair (Table 1) which can amplify JE NS3 region (9) and confirmed that it works on Japanese, Chinese, Thai, Malay, and Vietnamese JE virus isolates.

Cerebro-spinal fluids (CSF) from 17 Japanese JE patients were examined by PCR and ELISA. Only one case out of 17 patients showed PCR positive before sero-conversion measured by IgM-capture ELISA. On the contrary, sero-conversion was already observed in the 15 cases of PCR negative patients when the patients showed the apparent encephalitis symptoms. It is supposed that the virus in CSF had been cleared at that time. Thus, IgM-capture ELISA is a realistic tool for rapid diagnosis of JE rather than PCR.

5. Conclusion

Polymerase chain reaction greatly facilitates quick detection and identification procedure on dengue and JE virus. Especially, direct RT-PCR is the most rapid diagnostic tool in dengue acute phase prior to the elevation of anti-dengue IgM and IgG antibodies.

However, as viremia is cleared quickly after specific antibodies are elicited, both PCR and ELISA diagnosis are necessary. On the other hand, PCR is not an effective diagnostic tool for Japanese encephalitis (JE) as far as we have examined.

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