Comparison of Two Different Methods for the Purification of Polymerase Chain Reaction (PCR) Products Used in Direct Sequencing, in an Applied Biosystems 373A DNA Sequencing System

Ravindran THAYAN[1,2], Kouichi MORITA[2], Balasubramaniam VIJAYAMALAR[1], Tong Keong CHEW[1], Mangalam SINNIAH[1] and Akira IGARASHI[2]

[1]*Division of Virology, Institute for Medical Research, Jalan Pahang 50588, Kuala Lumpur, Malaysia*
[2]*Department of Virology, Institute of Tropical Medicine, Nagasaki University, 1−12−4 Sakamoto, Nagasaki, Japan 852*

Abstract: This study was carried out to compare the efficiency of two different purification methods used for removing excess primers and short oligonucleotides from PCR products. Our aim was to sequence PCR product directly. The two different purification methods were microfilter spin unit system (Millipore) and Quick Spin Column (Boehringer Mannheim Biochemica). The results indicated that the purification method using microfilter spin unit system was better and yielded better suquencing pattern compared to quick Spin Column.

Key Words: Dengue Virus, PCR product, Direct Sequencing Nucleotide

INTRODUCTION

Dengue infection (ranging from mild to severe cases) caused by the dengue virus, a mosquito-borne virus belonging to the flaviviradae family, has become a serious health problem in much of the tropical world (Halstead, 1965, 1980). The dengue viruses can be classified into four antigenically distinct serotypes. A more severe form of the disease, involving vascular and hemostatic abnormalities (dengue haemorrhagic fever-dengue shock syndrome, DHF-DSS), is responsible for a high mortality rate, primarily among children (Lanciotti, *et al.*, 1992).

It has been postulated that pathogenesis of severe form of dengue (DHF-DSS) is due to the virulence of the viral strain (Rosen, 1977). Sequencing data of viral genomes of numerous isolates have to be compiled to correlate genetic variety and phenotypic variation.

DNA sequencing methods have been around for several years, but only since the late 1970s has rapid and efficient nucleic acid sequencing has been possible (Brown, 1990). The two approaches often used in DNA sequencing are both sequencing of cloned polymerase

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chain reaction (PCR) products and direct sequencing of the PCR products. The advantages of direct sequencing of PCR products over conventional sequencing of cloned, single-stranded DNA are manifold. Time-consuming creation and screening of libraries, fragment purification, subcloning and plasmid preparation steps are all eliminated thus saving time and reducing costs of the process (Meltzer, 1993).

In this study we evaluated two methods of PCR product purification to further cut down the steps required for sequencing. The target viral RNA fragment is the envelope / nonstructural protein 1 (E/NS1) junction comprising of 240 nucleotides. This area of genome was chosen because it showed a uniform rate of random mutation with no hypervariable regions (Rico-Hesse, 1990).

MATERIALS AND METHODS

Virus isolates: The dengue virus was isolated from patients by inoculating serum samples from patients during the acute stage of the disease into C6/36 cell-line (Igarashi, 1978). Infected fluids were harvested 1 week after incubation at 28℃ and stored at −80℃ before use.
Primers: Primers used in this study were synthesized using an Applied Biosystems DNA synthesizer (Model 380B). The nucleotide sequence of the primers and the references are listed on Table 1.
Amplification of dengue virus RNA: Reverse transcriptase-PCR was performed as described by Morita *et al.*, (1991). The resulting PCR product was subjected to agarose gel electrophoresis and amplified DNA fragments were identified by ethidium bromide staining.
PCR product purification: PCR product used for direct sequencing have to be purified from excess primers and short oligonucleotides. Two methods were used for this purpose which were microfilter spin unit system (Millipore's Ultrafree MC filter, 30,000 NMWL, USA) and Quick Spin Columns Sephadex G 50, Fine (Boehringer Mannheim Biochemica, Germany). For the first method, PCR product was dispensed into the upper part of the tube and centrifuged at 7000 rpm for 5 minutes. The filtrate containing excess primers and short oligonucleotides was discarded and DNA (which remains on the filter) was washed three times with 200 microliters of TE buffer (pH 8.0). After the final washing, DNA was suspended in TE buffer. The amount of final DNA concentration was quantitated using optical density at 260 nm and stored at −20℃ before use. For the second purification method using Quick Spin Columns, the column was placed in a collection tube and centrifuged at 1500 rpm for 4 minutes to drain the buffer. Then PCR product was added into the column while holding it in an upright position after which it was placed into a second collection tube and centrifuged at 1500 rpm for 4 minutes. The elute in the second collection tube should contain the purified sample.
Sequencing: Purified PCR products were used as template for terminator cycle sequencing using Taq dyedeoxy terminator cycle sequencing kit (Applied Biosystems). The primers used in this step were similar to the primers used during the PCR amplification. The sequence reaction was purified of excess dyedeoxy terminators by using cetyl-trimethyl ammonium

bromide (CTAB) precipitation. Polyacrylamide gel electrophoresis was carried out using the Applied Biosystems DNA Sequencer Model 373A.

RESULTS

RT-PCR: Since sequencing was limited to Dengue 3 infected fluid, RT-PCR was carried out using dengue 3 type specific primers. The position of the target E/NS1 region, a 240-base pair(bp) sequence, is at 2217 till 2454. The sequences of the primers used in this study were generated from published data (Table 1). Dengue 3 type specific primer set produced a PCR product of 500-bp (Osatomi, K and Sumiyoshi, H. 1990).

Purification of PCR products: We have found that microfilter spin unit system (Millipore) removed the excess primers and short oligonucleotides more effectively than Quick Spin Column which still had excess primers (Figure 1).

Terminator cycle sequencing: We have also found that sequencing data obtained by using microfilter spin unit-purified PCR product yielded better pattern compared to Quick Spin Column-purified PCR products (Figure 2). We found that the method used successfully sequenced a DNA fragment of at least 400-bp, covering well the target region of 240 nucleotides.

Table 1. Nucleotide sequences of dengue virus primers

Primer*	Sequence (5' to 3')	Position	Reference**
D3S	ATTGGAGACAAAGCCCTGAA	2072−2091	1
D3C	TCCATTCTCCCAAGCGCCTG	2572−2553	2

* −S, sense primer; C, complementary primer; Dengue virus type 3
** −1 & 2− Osatomi, K & Sumiyoshi, H. (1990)

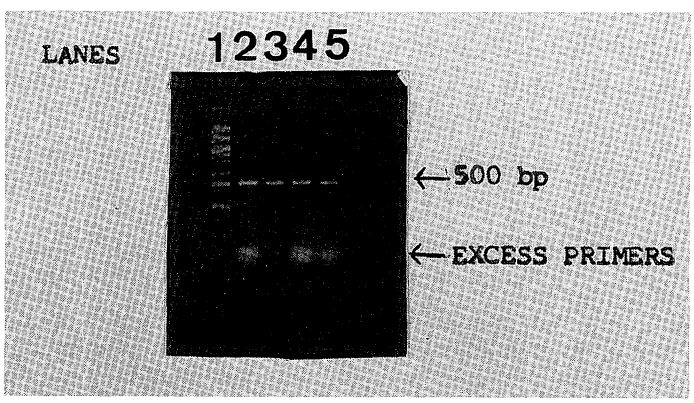

Fig. 1. Agarose gel (3.0% Nusieve 3:1) of PCR products. Lane 1, Phy DNA marker; lane 2 & 3, PCR product before and after microfilter spin unit purification; lane 4 & 5, PCR product before and after Quick Spin Column purification.

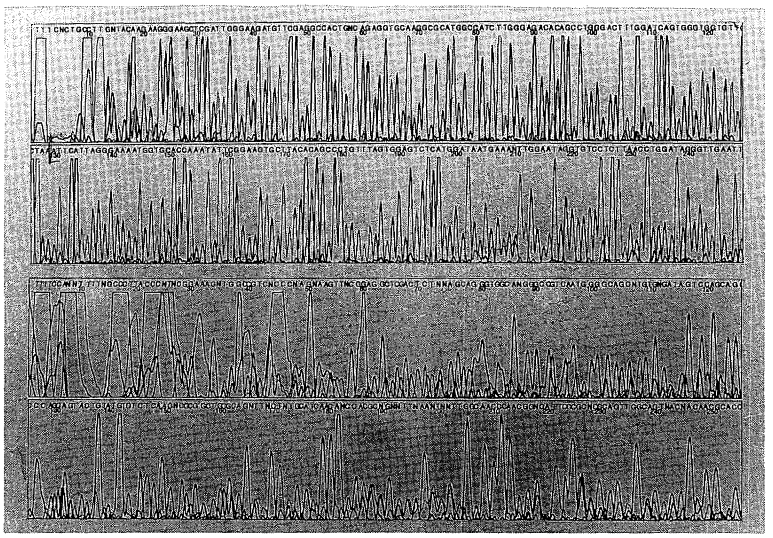

Fig. 2. Comparison of sequence pattern between two purification methods. The top pattern is for microfilter spin unit system purification while the bottom pattern is for spin column purification.

DISCUSSION

DNA sequencing is probably the most important tool available to the molecular biologist by which the precise order of nucleotides in a piece of DNA can be determined (Brown, 1990). In this study we had carried out direct sequencing of PCR products as this approach involves fewer steps, thereby, the faster and cheaper sequence data is obtained. It was found that the PCR product used in the sequencing was very well purified by using the microfilter spin unit system (Millipore) resulting in good sequence data. However the sequence data of PCR product, purified using Quick Spin Column was not satisfactory. This, was probably due to the need of careful manipulation of steps which are crucial when the columns were being used. The important concern in direct sequencing of PCR product is probably the quality of PCR product used as template. If the PCR product is pure and free from mispriming or nonspecific amplification products, clear sequencing ladders will usually result (Meltzer, 1993). This study provides a sound base for further sequencing analysis of Malaysian dengue isolates.

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