应用PCR快速制备细小病毒B19诊断芯片探针

Human parvovirus B19 is the etiologic agent of erythema infectiosum (EI) and transient aplastic crisis (TAC) in patients with hemolytic anemias, and has been identified to associate with fetal death, arthritis, and chronic anemia[1].

In China, cases of human parvovirus B19 infection were initially identified in the year 1990, and numerous reports pertaining to the infections came forth ever since. As this virus poses serious threat to public health, a simple and sensitive method for its detection is urgently desired[2].

Antibody capture assay for human parvovirus B19 immunoglobulin (IgM) antibodies has been generally acknowledged as a practical way to detect acute infection of this virus. In patients with chronic infection, however, antibody assays are usually not sufficient for the diagnosis [3]. Persistent infection may occur with or without the presence of IgG and/or IgM antibodies and can be shown only by detecting the virus over time.

The technique of DNA microarray provides a possible alternative for detecting the infection of human parvovirus B19, which is relatively simple and sensitive, and much more efficient. Compared with traditional diagnostic techniques, it possesses obvious advantages for its high degree of integration, micromation and automation[4][5]. In this present study, we prepared human parvovirus B19 microarray probes using PCR, and explored the feasibility of this method for developing DNA microarray probes.

MATERIALS AND METHODS

Plasmid

The plasmid harboring human parvovirus B19 was provided by Dr Laura Kakkola, Department of Virology, Haartman Institute, University of Helsinki, Finland.

Chemicals and Reagents

Premix Taq, dNTP, EcoRⅠ, ApaLⅠ , pMD18-T vector, T4 DNA ligase, plasmid miniprep kit were products of Takara Biotechnology Co. Ltd (Dalian). PCR primers were synthesized by BIOASIA Biotech- nology Co. Ltd.

Bacterial strains

E.coli strain XL-1 used in the experiments was maintained in our laboratory.

Identification of the plasmid

After the digestion with the restriction endonu- cleases ApaLⅠ and EcoRⅠ, the

plasmid containing human parvovirus B19 was transferred to 20 μl ApaL Ⅰ digestion system. After resuspension, the solution was incubated for 4 h at 37 ℃. Following precipitation with 50 μl ice-cold ethanol and treated for 30 min at -80 ℃, the nucleic acids centrifuged at 12 000 r/min at 4 ℃ for 15 min to result in small pellets, which were rinsed with 75% (V/V) ice-cold ethanol, air dried, loaded into 20 μl EcoR I digestion system, resuspended, and then incubated for 4 h at 37 ℃.

The nucleic acid was rinsed likewise and then dissolved in 5 μl sterilized DEPC-treated water, with 3 μl of the sample subsequently loaded onto 1.5% agarose gel for electrophoresis at 64 V for 1 h followed by photography under UV light.

Primers

We designed 12 different pairs of primers to amplify the fragments of the 3 genes that encode the proteins NS1 (relative molecular weight of 77 000), VP1 (84 000), and VP2 (58 000), respectively. The locations of the 12 different PCR primer pairs in the B19 genome and size of the amplified fragments as expected are shown in Tab1. The bases were numbered according to the DNA sequence deposited in the GenBank.

PCR amplification

One microliter of plasmid was added to the PCR mixture containing 50 μl 2×premix (PCR buffer, dNTPs, Taq polymerase, Mg^{2+}), 1 μl sense primer and 1 μl antisense primer (0.25 μmol/L each), pre-denatured at 95 ℃ for 3 min before 30 cycles were performed in the sequence of 94 ℃ for 45 s, 55 ℃ for 60 s and 72 ℃ for 90 s. The amplified products were subjected to electrophoresis on 1.5% agarose gel and ethidium bromide staining.

Ligation and transformation

After purification with a PCR product purification kit, the PCR products were inserted into pMD18-T vector. The ligation mixture containing 4 μl PCR products, 1 μl pMD18-T vector and 5 μl ligation buffer was incubated at 16 ℃ for 4 h followed by transformation of 100 μl E.coli XL-1 competent cells. Following shaking for 45 min at 37 ℃, 100 μl of the transformed cells were plated on an agar dish containing Amp, X-Gal and IPTG, and incubated for approximately 15 h at 37 ℃.

The white clones containing target frag- ments were selected and identified with pMD18-T vector primers (primer A 5'-CTAAAACGACGGCCA GT-3', primer B 5'-CAGGAAACAGCTATGAC-3'), the positive clones undergoing plasmid extraction and se- quencing of inserted fragments.

Blasting

The sequenced DNA fragments were blasted with GenBank data to align and identify the fragments.

RESULTS

Restriction endonuclease digestion

Annhyb4 analysis shows that 6 fragments were resulted after restriction endonucleases

ApaL Ⅰ and EcoR Ⅰ digestion, with lengths of 3 570, 1 740, 1 221, 970, and 550 bp respectively. The result of agarose gel electrophoresis is shown in Fig.1, which demonstrates the agreement of the result with previous theoretical expectation.

Tab.1 Locations of the 12 primer pairs in B19 genome

Set	Primer pair	Primers location	Product length (bp)
1	P1, P2	(2878-2901) (3104-3080)	227
2	P3, P6	(1578-1601) (1861-1838)	284
3	P4, P6	(1678-1704) (1861-1838)	184
4	P5, P6	(1755-1779) (1861-1838)	107
5	P7, P10	(3323-3344) (3768-3746)	446
6	P8, P10	(3439-3460) (3768-3746)	330
7	P9, P13	(3746-3768) (4416-4393)	671
8	P11, P13	(4306-4327) (4416-4393)	111
9	P11, P15	(4306-4327) (4868-4844)	563
10	P11, P16	(4306-4327) (5003-4982)	698
11	P12, P16	(4393-4416) (5003-4982)	611
12	P14, P16	(4844-4868) (5003-4982)	160

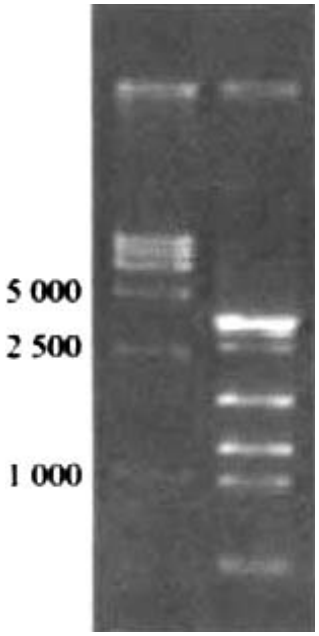

Fig.1 Aarose gel (1.5%) electrophoresis after restriction endonuclease digestion of the plasmid harboring the human parvovirus B19 genome

PCR amplification

The results of agarose gel electrophoresis demonstrated that amplification with the 12 pairs of primers yielded 12 fragments of the human parvovirus B19 DNA with the sizes as expected (Fig.2).

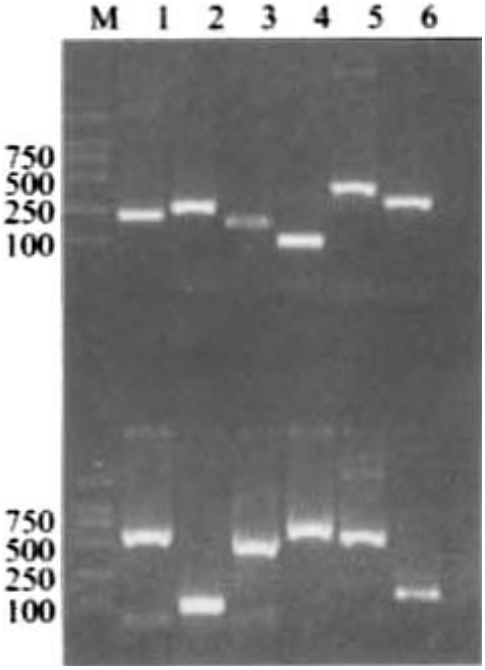

Fig.2 Agarose gel electrophoresis of the 12 PCR products

Sequence analysis

Blast with GenBank database showed that the sequences of the PCR products conformed to human parvovirus B19 genome fragment sequence located at B19 virus isolate 2_980414 VP1 protein gene 264-453 (Fig.3).

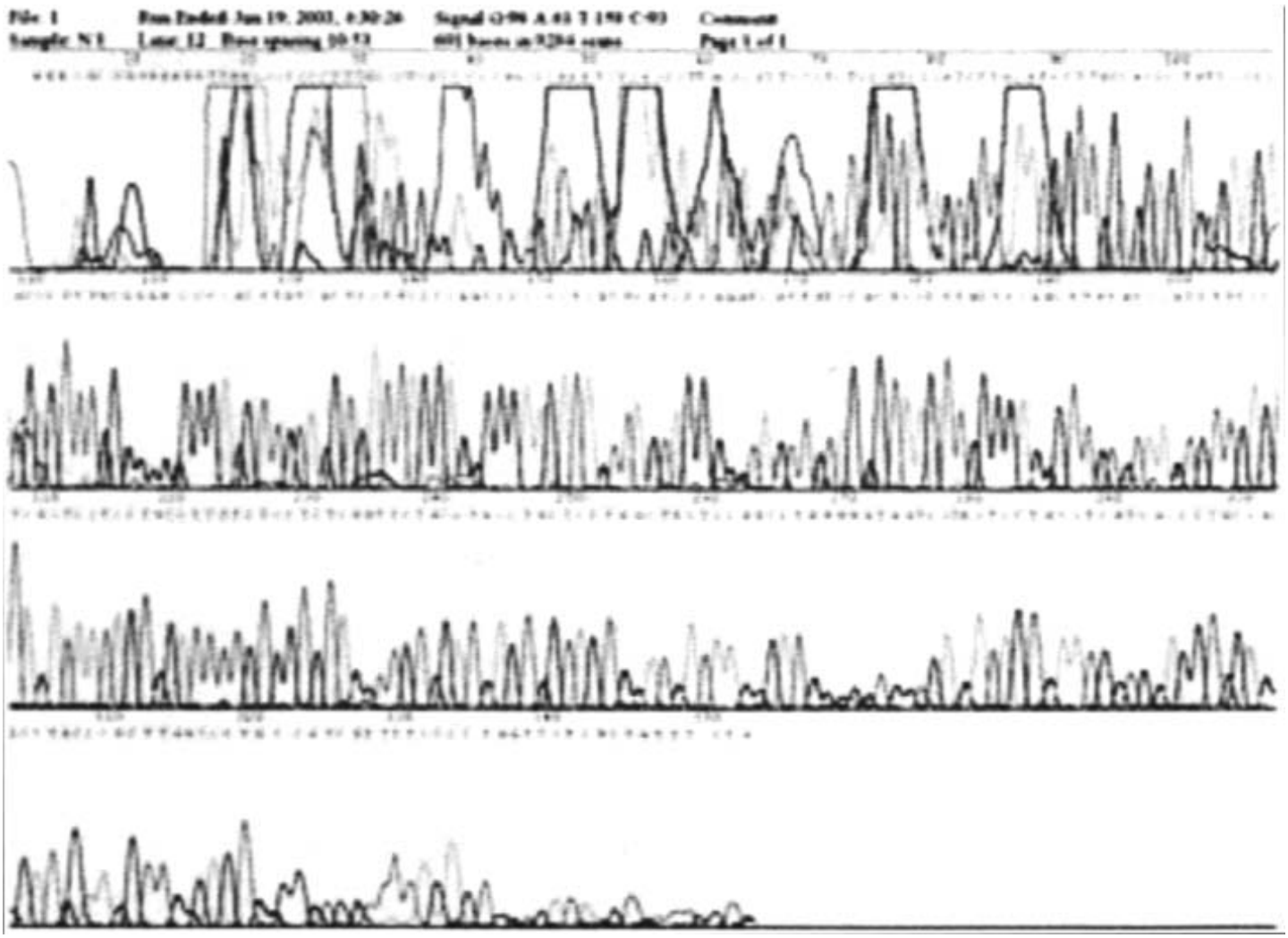

Fig.3 Sequence analysis of one of the DNA fragments from human parvovirus B19

DISCUSSION

Human parvovirus B19 replicates only in the erythroid precursor cells derived from the bone marrow. Its infection is frequently asymptomatic (in 20%-50% of the cases) and therefore an accurate diagnosis can only be made by serological testing[6].

As the virus antigen detection and its IgM and IgG antibody determination using enzyme immunoassays are not reliable for diagnostic purposes and lack specificity, its DNA detection in the amniotic fluid, fetal blood, ascitic fluid, and fetal biopsies or placenta specimens seems to be a better alternative[7].

The DNA microarray system has been shown to provide good diagnostic sensitivity and specificity in the diagnosis of human parvovirus B19[4], and with this system, all the genomic information of the virus can be integrated into a microarray of merely 1 cm2 in size, which offers dependable basis for the clinical diagnosis and medication.

Currently two methods for preparing the DNA microarray probe are employed[5], one is PCR amplification of the DNA fragments, the other, artificial synthesis of the oligonucleotide fragments. The former method was adopted in this study, and with Primer Premier 5.0, special primers in 16 different pairs were designed that located within the conservative regions of NS1, NS2, and VP2. Due to the convenience of PCR, we could easily harvest the DNA microarray probes after purification of the PCR products.

Probe preparation by PCR is effective for low probe density DNA microarray, as the one used in this study. When high-density DNA microarray is demanded for assay of complicated genome DNA, large amount of probes are needed, which can be achieved with improved restriction display technique[5][6] that yields gene fragments as probes in sufficient quantity with adequate and homogeneous sizes within a short period of time.

Using this technique, the DNA microarrays have been successfully constructed for detection of human immunodeficiency virus, tumor-related genes, and the study of spermatid gene expression profile. This method can also be used to label samples with fluores- cence for subsequent DNA microarray hybridazition, and a number of experiments have produced good results[8][9][10].

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回结果列表