DESIGN OF DEGENERATE PRIMERS FOR MULTIPLEX NESTED-PCR DETECTION OF HUMAN LYMPHOTROPIC HERPESVIRUSES

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Abstract. To develop the rapid diagnosis and typing of human lymphotropic herpesviruses by using multiplex nested-PCR, the primary PCR (1º PCR) primers were redesigned as degenerate primers based on a highly conserved sequences of each DNA polymerase gene of EBV, CMV, HHV-6, HHV-7 and HHV-8. The forward degenerate primer (HHV/1+) contained 12 different sequences, whereas there were 8 different sequences in the reverse degenerate primer (HHV/1-). Optimization of multiplex nested-PCR assay conditions were performed to search for the appropriate amount of degenerate primers, dNTP, *Taq* DNA polymerase, template of secondary PCR (2º PCR) and annealing temperature used in 1º PCR reaction. Detection sensitivity was the same as described in previous report (approximately 10-100 genome copies). To ensure a true negative result, PCR detection of hepatitis B virus genome was used as internal control. Our presented results, the designed degenerate primers could be used to detect various types of HHV by multiplex nested-PCR.

INTRODUCTION

Epstein-Barr virus (EBV, HHV-4), human cytomegalovirus (CMV, HHV-5), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7) and human herpesvirus 8 (HHV-8, Kaposi's sarcoma associated herpesvirus) are known human lymphotropic herpesvisus (HHVs) whose natural host is human. Although the viruses are different from one another, regarding their biological behavior and genomic arrangements, they share the same ability to establish latency after primary infection (Roizmann *et al*, 1992). Recurrent or reactivated HHVs infection are commonly found as opportunistic diseases in HIV-infected person (Fabio *et al*, 1997; Schulz, 1998; Clark, 2000) or in immunosuppressed patients following bone marrow, kidney, liver, or heart transplantation (Chan *et al*, 1997; Osman *et al*, 1997; Clark, 2000). HHV-6 and HHV-7 have also been associated with febrile illness and childhood diseases, exanthem subitum (roseola infantum) (Yamanishi *et al*, 1988; Tanaka *et al*, 1994). Nevertheless, EBV appear to be an important etiological factor for nasopharyngeal carcinoma. The use of serum/plasma EBV DNA as a reliable tumor marker prior to, during, and after treatment of the cancer was reported (Liebowitz *et al*, 1994; Shotelersuk *et al*, 2000).

Recently, PCR-based assays have been recognized as sensitive and specific method for molecular detection and identification of HHVs (Wakefield *et al*, 1992; Tenorio *et al*, 1993; Vandevanter *et al*, 1996; Clark *et al*, 1997; Kidd *et al*, 1998; Minjolle *et al*, 1999; Pozo and Tenorio, 1999; Johnson *et al*, 2000; Kessler *et al*, 2000; Kearns *et al*, 2001). A multiplex nested-PCR for simultaneous detection and typing of HHV4 (EBV), HHV-5 (CMV), HHV-6, HHV-7 and HHV-8 was developed by Francisco Pozo and Antonio Tenorio in 1999. Two sets of specific primers, designed for amplification of a highly con-

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served region within the DNA polymerase gene, were used in the first and the second rounds of nested-PCR. According to multiplex nested-PCR assay, specific primers used in the primary PCR (1º PCR) reaction were derived from conserved sequences alignment of each herpesviruses DNA to amplify the same size (194 bp) of PCR products. Then specific primers of the secondary PCR (2º PCR) reaction were designed from different region within 194 bp of first round PCR products in order to produce DNA of different size. It is noteworthy that the sequence alignment of each of the 5 forward and the 5 reverse primers in the 1º PCR reaction were quite the same. For reduction of the cost of the primer, a pair of degenerate primers should be designed to use instead of the specific ones.

In this report, we described the degenerate primers design for multiplex nested-PCR of all HHV 4 - HHV 8 typing. Optimum conditions including appropriate amount of degenerate primers, dNTP, *Taq* DNA polymerase, template of 2º PCR and annealing temperature used in 1º PCR reaction were investigated. In order to detect the presence of DNA polymerase inhibitors or reaction failures, PCR amplification of a hepatitis B virus (HBV) DNA fragment was applied as internal control. Moreover, the sensitivity of the PCR assay was estimated by testing the dilution of HHV DNA (194 bp) including recombinant plasmid.

MATERIALS AND METHODS

Degenerate primers designation

As previously reported by Pozo and Tenorio in 1999, forward and reverse primer sequences in the 1º PCR amplification of HHV DNA polymerase gene were aligned (Table 1). A pair of degenerate primers were then designed and synthesized as HHV/1+ (5′ GTCATTTATGG BGAYACKGA 3′) and HHV/1- (5′ ATCC CCATGTATCKYTTYTT 3′), when B = C or G or T, Y = C or T, K = G or T. For 2º PCR reaction, the specific primers based on a report of Pozo and Tenorio (Table 2) were used for

Table 1
Degenerate primers designation.

Forward primer sequences									
HHV-6	5′	GTA	ATT	TAT	GGT	GAT	ACG	GA	3′
HHV-7	5′	GTG	ATT	TAT	GGT	GAT	ACT	GA	3′
CMV	5′	GTC	ATC	TAC	GGG	GAC	ACG	GA	3′
EBV	5′	GTC	ATC	TAC	GGG	GAC	ACG	GA	3′
HHV-8	5′	GTC	ATA	TAC	GGC	GAC	ACT	GA	3′
					↓				
HHV/1+	5′	GTC	ATT	TAT	GGB	GAY	ACK	GA	3′
Reverse primer sequences									
HHV-6	5′	AAA	AAA	AGA	TAC	ATT	GGT	AG	3′
HHH-7	5′	AAG	AAA	CGT	TAC	ATT	GGA	AA	3′
CMV	5′	AAG	AAA	CGT	TAC	ATC	GGC	AA	3′
EBV	5′	AAG	AAG	AGA	TAT	GTG	GGG	GT	3′
HHV-8	5′	AAA	AAG	AGA	TAC	GTG	GGG	GT	3′
					↓				
	5′	AAR	AAR	MGA	TAC	ATG	GGG	AT	3′
HHV/1-	3′	TTY	TTY	KCT	ATG	TAC	CCC	TA	5′

B = C or G or T Y = C or T K = G or T
R = A or G M = A or C

Table 2
Primer sequences for specific typing of HHV-4 - HHV-8.

Primers	Sequences (5′-3′)								T_m
HHV/1+	GTC	ATT	TAT	GGB	GAY	ACK	GA		50-54
HHV/1-	ATC	CCC	ATG	TAT	CKY	TTY	TT		44-48
EBV/2+	ACC	CGG	AGC	CTG	TTT	GTA	GC		64
EBV/2-	GGA	GAA	GGT	CTT	CTC	GGC	CTC		68
CMV/2+	GGG	CCC	AGC	CTG	GCG	CAC	TA		70
CMV/2-	GAC	GAA	GAC	CTT	TTC	AAA	CTC		60
HHV6 (A/B)/2+	GCC	AAA	CAT	ATC	ACA	GAT	CG		58
HHV6 (A/B)/2-	GGA	CAT	AAA	ATC	TTY	TCR	AAC	TC	60
HHV7/2+	GTT	ACT	TTC	AAA	AAT	GTT	TGT	CCC	64
HHV7/2-	GGA	AAT	AGG	ATC	TTT	TCA	AAT	TC	60
HHV8/2+	GGA	CAG	CGT	GTC	AGA	CTT	CG		64
HHV8/2-	CTT	GAA	GAT	CTT	TTC	AGC	CTC		60

T_m = Melting temperature
= 2 x (A + T) + 4 x (G + C)
B = C or G or T, Y= C or T, K = G or T

amplification of different HHV fragments (EBV = 54 bp, HHV-6 = 68 bp, CMV = 78 bp, HHV-8 = 97 bp and HHV-7 = 122 bp).

DNA extraction of HHVs

All HHVs strains were isolated from clinical specimens. EBV was obtained from patients with nasopharyngeal carcinoma, CMV and HHV-8 from opportunistic infections in liver transplant patients and HIV-infected patients, respectively. HHV-6 and HHV-7 were generously provided from Dr Kruavan Balachandra, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand.

Each DNA of HHVs was extracted from clinical specimens (serum 50 μl), based on proteinase K-phenol-chloroform extraction procedure (Sambrook *et al*, 1989). After removing denatured protein, DNA was precipitated by adding 0.3 M sodium acetate, 60 μg glycogen and absolute ethanol, then kept at -20ºC for an hour. Pellet DNA was obtained by centrifugation at 13,000 rpm for 15 minutes. The pellet was then twice washed with 70% ethanol and air-dried. The DNA pellet was dissolved in 10 μl of sterile distilled water and aliquot 5 μl into 45 μl of PCR reaction mixture.

Optimum conditions for multiplex nested-PCR assay using degenerate primers

In order to generate a HHVs typing via multiplex nested-PCR assay with degenerate primers, PCR reaction mixtures were performed, quite similar as ones previously described by Pozo and Tenorio (1999). The difference was in 1º PCR step, where the degenerate primers were employed instead of specific primers and the amplification at appropriate annealing temperature was based on Tm of degenerate primers. In detail, a 1º PCR reaction mixture of 50 μl in total volume was prepared containing 5 μl of viral DNA extraction, 20 pmol of degenerate primers (Gibco, Life Technologies, ML, USA), 200 μM of each dNTPs (Perkin-Elmer, NJ, USA), 1.25 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany), 4 mM of $MgCl_2$ and 1X PCR buffer. Amplification was carried out on a Thermal Cycler 2400 (Perkin-Elmer, NJ, USA). An initial denaturation step at 94ºC for 4 minutes was followed by 30 cycles consisting of 30 seconds at 94ºC, 1 minute at 45ºC and 30 seconds at 72ºC. A final extension step at 72ºC was carried out for 10 minutes. In the 2º PCR, 3 μl of 1º PCR product was added to 47 μl of reaction mixture, consisting of 10 pmol of each specific primer (Pozo and

Tenorio, 1999), 200 μM of each dNTPs, 1.25 U of *Taq* DNA polymerase, 2 mM of $MgCl_2$ and 1X PCR buffer. Amplification was performed by applying the same conditions used in the 1º PCR step, except the annealing temperature was 47ºC. To detect a false negative PCR reaction, amplification of known hepatitis B virus (HBV) DNA was used as internal control. A 10 μl of each 2º PCR product was subjected to electrophoresis on a 3.5% agarose gel (Invitrogen, Groningen, Netherlands) in 0.5X TBE buffer at 90 DC voltage. DNA was stained with ethidium bromide and was then detected by ultraviolet transilluminator (Gel Doc 1000, Bio-Rad, CA, USA).

In order to optimize recent multiplex nested-PCR assay by using degenerate primers, variant effective factors were investigated. For 1º PCR step, appropriate annealing temperature (40º or 45ºC) and amount of degenerate primer (10 or 20 or 30 pmol) were studied. Three effective factors of 2º PCR step were also tested, *eg* volume of 1º PCR product (1 or 3 or 5 μl), amount of dNTP (200 or 300 or 400 μM) and amount of *Taq* DNA polymerase (1.25 or 2 or 2.5 U). Each PCR reaction was employed as standard assay described above, except only one factor was changed. From investigations, optimal condition for this assay was concluded as follows:

1º PCR : Viral DNA extraction 5 μl
(50 μl) Each degenerate primer 20 pmol
dNTP 200 μM, *Taq* DNA polymerase 1.25 U
$MgCl_2$ 4 mM , 1X PCR buffer
PCR cycle:
94ºC 4 minutes 1 cycle
94ºC 30 seconds/45ºC 1 minute/72ºC 30 seconds 30 cycles
72ºC 10 minutes 1 cycle

2º PCR: 1º PCR product 3 μl
(50 μl) Each specific primer 10 pmol
dNTP 300 μM , *Taq* DNA polymerase 2 U
$MgCl_2$ 2 mM , 1X PCR buffer
PCR cycle : The same as 1º PCR cycle
Exception : Use annealing temperature 47ºC instead of 45ºC

Sensitivity of the assay

Each HHVs DNA (194 bp) which was amplified by PCR was cloned into *Eco*R I / *Bam*H I site of pUC19 (BioLab, CA, USA). Recombinant plasmids were transformed into *E. coli* DH5α-competent cells. Tranformants were then selected on LB / ampicillin / IPTG / X-gal plates. And each plasmid DNA was purified via QIA prep spin Miniprep kit (Qiagen, Hilden, Germany). The plasmid concentrations were estimated spectrophotometrically at A_{260}.

To determine the sensitivity of the PCR assay, each stock of plasmid DNA at 10^{10} copies/μl was diluted in 100-fold steps to 10^2, and thereafter in 10-fold steps to 1 copy/μl. Variant amounts of plasmid DNA of 1, 10, 100, 1,000 and 10,000 copies were used as DNA templates in each 1º PCR reaction that contained degenerate primers (HHV/1+ and HHV/1-), following with 2º PCR amplification as described above. Assay sensitivity was defined as the minimal number of copies of DNA templates whose PCR products could be detected via agarose gel electrophoresis procedure.

RESULTS AND DISCUSSION

PCR-based assay is widely used in clinical laboratory for diagnosis of many organisms including HHVs. A multiplex nested-PCR assay for typing HHVs, *eg* EBV, CMV, HHV-6, HHV-7 and HHV-8, was reported in 1999 by Francisco Pozo (Pozo and Tenorio, 1999). According to the procedure, a total of 20 specific primers were designed. The 10 specific primers of the 1º PCR reaction produced the same size (194 bp) of PCR products, whereas another 10 specific primers of the 2º PCR reaction produced another size of DNA fragments for HHVs diagnosis. Because each 5 forward and 5 reverse primers of 1º PCR reaction derived from conserved sequences alignment of each HHVs DNA polymerase gene, therefore, the sequence of the primers were quite the same. The notify led to save cost via using degenerate primers instead of specific primers in 1º PCR reaction. A pair of degenerate primer was newly designed with forward primer (HHV/1+), containing 12 different sequences (T_m ~50-54ºC), whereas, reverse primer (HHV/1-) consisted of 8 different sequences (T_m ~44-48ºC).

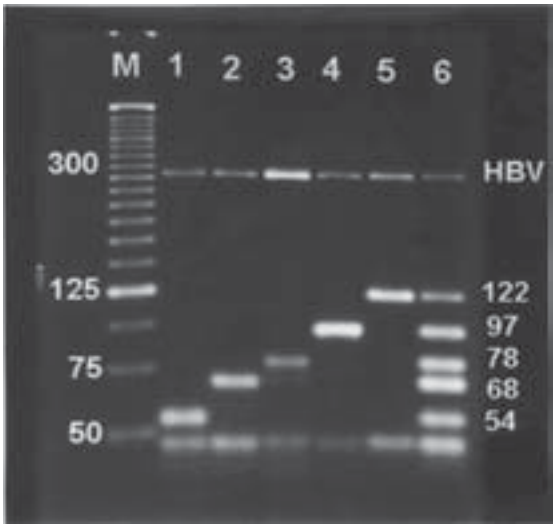

Fig 1–Gel electrophoresis of PCR products and multiplex PCR products of EBV (54 bp), HHV-6 (68 bp), CMV (78 bp), HHV-8 (97 bp), HHV-7 (122 bp) and internal control HBV-DNA (~300 bp). M = Marker, lane 1; EBV, lane 2; HHV-6, lane 3; CMV, lane 4; HHV-8, lane 5; HHV-7 and lane 6; multiplex nested PCR HHVs.

Using degenerate primers, it effect not only annealing temperature but also amount of degenerate primers in 1º PCR reaction. The appropriate annealing temperature of degenerate primers is 45ºC, comparing to 53ºC of specific primers. Opposite to the annealing temperature, double of degenerate primers (20 pmol) is an optimum condition. And 3 μl of 1º PCR product is suitable template for 2º PCR amplification. In order to amplify all HHVs DNA fragments in 2º PCR reaction, 300 μM of dNTP and 2 U of *Taq* DNA polymerase were also recommended. According to the optimum condition, each HHVs was differentiated from each others as shown in Fig 1. PCR products of 54 bp for EBV, 68 bp for HHV-6, 78 bp for CMV, 97 bp for HHV-8, 122 bp for HHV-7 and ~300 bp for HBV (internal control) were detected. The sensitivity of the assay was approximately 10-100 genome copies, similar to a previous report (Pozo and Tenorio, 1999) (Fig 2).

From our results, the designed degenerate primers could be used for detection of HHVs

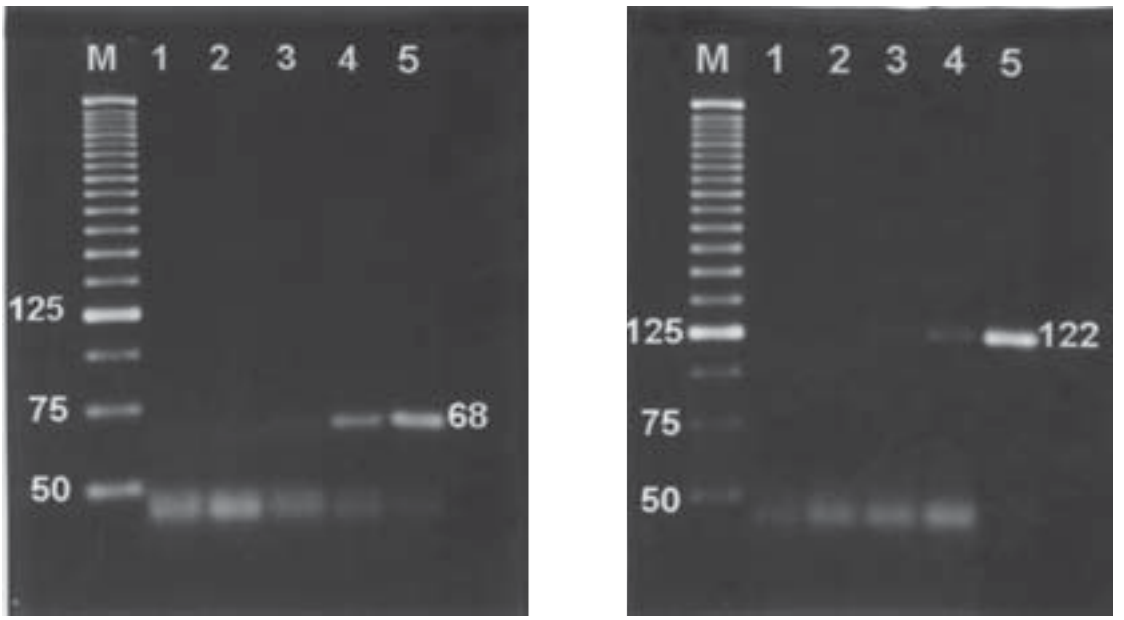

Fig 2–Sensitivity of multiplex-nested PCR assay using degenerate primers for HHVs detection was equivalent to 10 - 100 copies viral DNA. Left : HHV-6 and Right : HHV-7.

via multiplex nested-PCR with no significant difference from the one that used specific primers. The method would be useful for rapid detection and typing HHVs in clinical specimens.

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