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Determination of HIV-1 Subtypes (A–D, F, G, CRF01_AE) by PCR in the Transmembrane Region (gp41) With Novel Primers

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HIV-1 has a huge genetic diversity. So far, nine subtypes have been isolated, namely, subtypes A, B, C, D, F, G, H, J, and K. Epidemiological study provides information which may help in the development of HIV-1 prevention programs or health policies. In the future, subtyping may also be critical for vaccine development, and an effective anti-viral drug will need to be effective for different subtypes of HIV virus. The analysis of the nucleotide sequence of the v3 region is considered the most reliable method for determining the HIV-1 subtype. However, the procedures for determining the v3 sequences are complicated and time consuming, requiring expensive reagents, equipment, and well-trained personnel. The polymerase chain reaction (PCR) method using subtype-specific primers for HIV-1 subtyping is easier and faster. The objective of this study was to develop subtype-specific primers for subtyping PCR. The specific primers were designed for subtypes A, B, C, D, F, G, and CRF01_AE, and these primers could be applied to assay for various HIV-1 subtypes in the clinical samples. The specific primers were designed for each subtypes in the gp41 region. The result of PCR was compared with the subtypes which was determined by the v3 sequence. The results of subtyping by PCR using the newly designed primers could detect 29 of 33 patients tested, and all matched those obtained by nucleotide sequencing of the env v3 region except for three subjects, which were differentiated as CRF02_AG. The newly designed primers functioned accurately and conclusively. In comparison with PCR as a method for the determination of subtypes, sequence analysis requires better-trained personnel, more expensive reagents, and more equipment and time. ***J. Med. Virol. 76:16–23, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HIV-1; subtyping; PCR

INTRODUCTION

HIV-1 has numerous genetic variations and can be divided by group, subtype, and subsubtype. The majority of HIV-1 strains belong to the M (Major) group, which causes pandemic HIV-1 infection. The O (Outlier) group and the N (Non-M/Non-O) group are seldomly found. Both the O and N groups have high genetic diversity from the M group [Charneau et al., 1994; Gurtler et al., 1994; Vanden Haesevelde et al., 1994; Loussert-Ajaka et al., 1995; Simon et al., 1998].

The M group has thus far been subdivided into nine isolated subtypes: A, B, C, D, F, G, H, J, and K [Carr et al., 1998; Robertson et al., 2000]. Although some isolates from Cyprus and Greece (94CY032, PVMY, and PVCH) were recognized previously as subtype I, they were shown to be a recombinant strain upon reanalysis. Subtype K was recognized initially as subtype F3, and later designated subtype K. Subtypes A and F are divided, respectively, into subsubtypes A1 and A2, and F1 and F2 [Triques et al., 1999, 2000]. Subtypes B and D are similar [Robertson et al., 2000; Triques et al., 2000], and although they should be reclassified as a single subtype, for the sake of historical consistency, they remain classified as different subtypes.

HIV-1 has been divided into not only many subtypes but also many circulating recombinant forms (CRFs), and so far 15 CRFs have been isolated [Carr et al., 1998]. For example, in the CRF01_AE strain, only the env region belongs to subtype E; the rest of the regions

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belong to subtype A, and the original full-length of subtype E is no longer found [Carr et al., 1996; Gao et al., 1996].

Worldwide, the incidence of HIV-1 subtypes is as follows: subtype C (47.0%), subtype A (27.2%), subtype B (12.3%), subtype D (5.3%), and CRF01_AE (3.2%) [Osmanov et al., 2002]. Certain subtypes prevail in distinct geographical areas, and different subtypes may predominate for each of the different means of infection [Essex, 1999]. In Thailand, subtype B is predominant among those infected through intravenous drug use, and subtype E among those infected through sexual intercourse [Ou et al., 1992, 1993; Weniger and Brown, 1996; Lole et al., 1999].

The gp120 of the HIV-1 envelope gene comprises five variable domains interspersed with conserved regions. The third variable (v3) region plays an important role in biological properties such as cell tropism, cytopathic effect, and pathogenicity [Robert-Guroff et al., 1994; Palker et al., 1988; Takahashi et al., 1988]. Therefore, analysis of the nucleotide sequence of the v3 region is considered as the most reliable method for determining the HIV-1 subtype. However, the procedures for determining the v3 sequences are complicated and time consuming, requiring expensive reagents, equipment, and well-trained personnel. For determining HIV-1 subtypes of a large number of samples, the heteroduplex mobility assay (HMA) and the peptide enzyme-linked immunosorbent assays (PELISAs) are usually used [Cheingsong-Popov et al., 1994; Wasi et al., 1995; Gaywee et al., 1996; Novitsky et al., 1996; Delwart et al., 1998]. Both of these methods often show cross-reaction or are not reliable. Recently, new methods were attempted to detect subtypes and recombinants [Hoelscher et al., 2002; Plantier et al., 2002]. However, these new methods are more difficult and complicated than PCR. Furthermore, the cost of the single PCR method in particular is approximately 10 times less than that of the sequence method, and thus consideration should be given to its preferential use in developing countries in the future [Yagyu et al., 2002].

The objective of this study was to develop subtype-specific primers for subtyping PCR, since the PCR method is easy, fast, cheap, and accurate. Therefore, specific primers were designed for subtypes A, B, C, D, F, G, and CRF01_AE, and these primers could be applied to examine various HIV-1 subtypes in clinical samples. This method could be applied with several advantages in developing countries, which face a large number of people living with HIV/AIDS.

MATERIALS AND METHODS

Clinical Specimens

Blood samples were collected from HIV-1 carriers with informed consent (Table I). All the subjects were positive originally for anti-HIV-1 antibody. Six samples were from Brazil and the patients had a history of treatment. Six samples from Japan, 2 samples from Kenya, 2 samples from Thailand, 1 sample from Tanzania, and 15 samples from Africa, for all of which a history of treatment was not available. The collection site of one sample was not specified nor the history of medication was known. Blood sample was obtained without any anti-coagulant and incubated at 56°C for 1 hr and stored at −20°C.

DNA Extraction

Stored samples were thawed at room temperature, centrifuged at 12,000*g*, and the supernatants were discarded. The cell pellets were suspended in 500 μl of DNA extraction buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA), 5 μl of 10% SDS and 5 μl of Proteinase K (25 mg/ml) were added, and incubated at 56°C for 1 hr. Following phenol and phenol/chloroform extractions, DNA was ethanol-precipitated at −85°C for 1 hr. After centrifugation at 12,000*g* for 15 min, DNA pellets were rinsed with ice-cold 70% ethanol and dried. The dried pellet in each tube was dissolved in 15 μl of distilled H_2O and stored at −20°C until use.

PCR of Cellular Beta-Actin

To examine the integrity of the DNA samples, cellular *beta-actin* gene was amplified as described previously with b-F and b-R primers [Yagyu et al., 2002] (Table II). The PCR products were subjected to 1% agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide.

Differentiation of Subtype B and E by PCR With Subtype-Specific Primers

Nested PCR with two sets of primers were carried out for differentiating subtypes B and E as described previously [Yagyu et al., 2002]. The primers were BECO5 and BECO3 for the first round and BE-ANCH, B-SPEC, and E-SPEC for the second round in PCR reaction (Table II). The PCR products were subjected to 1% agarose gel electrophoresis at 100 V for 30 min and stained with ethidium bromide.

Determination of Subtypes by PCR With Novel Designed Primers

The primers were BECO5 and BECO3 for the first round and BE-ANCH, B-SPEC, C-SPEC, E-SPEC, and F-SPEC for the second round in PCR reaction (Table II, Fig. 1). The primers were 5′D and 3′D, or 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the alternative second round PCR (Table II).

A reaction mixture was made by adding 5.0 μl of 10 × PCR buffer with 22.5 mM $MgCl_2$ (Roche Diagnostics, Indianapolis, IN), 0.3 μl of enzyme mix (Roche Diagnostics), 1.5 μl each of dATP, dCTP, dGTP, and dTTP (25 mM each), 1.5 μl each primer (33 pM each), 1.0 μl of template DNA solution and adding distilled H_2O, up to 50 μl. The cycle condition was 93°C for 1 min, 50°C for 1.5 min, and 72°C for 2 min, for 30 cycles.

The second round PCR products were subjected to 2% agarose gel electrophoresis at 100 V for 1 hr and stained with ethidium bromide.

TABLE I. Samples and Subtyping Results

Sample	Sex	Age	Sample taken in	HV+ from when	Symptom	The way of infection	Drug therapy	Sampling date	v3 sequence	PCR differentiate subtype B and E[b]	PCR with newly designed primer[c]	PCR with newly designed primer[d]	PCR with newly designed primer[e]
BRON01	M	34	Brazil	Sep/95	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON02	M	24	Brazil	Aug/95	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON03	M	54	Brazil	Nov/96	AC	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON04	F	31	Brazil	Aug/98	AC	Sexual	+	Nov/99	F	—[f]	F	—[g]	—[g]
BRON05	M	33	Brazil	Mar/97	AIDS	Sexual	+	Nov/99	B	B	B	—[g]	B
BRON06	M	36	Brazil	Mar/93	AIDS	Sexual	+	Nov/99	B	B	B	—[g]	B
YoD	—[a]	—[a]	Japan	—[a]	—[a]	—[a]	—[a]	—[a]	D	B	B	—[g]	D
OS01	F	—[a]	Tanzania	—[a]	—[a]	Sexual	—[a]	—[a]	D	B	B	—[g]	D
OS02	F	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	D	B	B	—[g]	D
OS03	F	—[a]	Thailand	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS04	F	—[a]	Thailand	—[a]	—[a]	Blood transfusion	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS05	M	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS06	—[a]	—[a]	—[a]	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS07	F	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS08	F	—[a]	Kenya	—[a]	—[a]	Sexual	—[a]	—[a]	CRF02 AG	CRF01 AE	CRF01 AE	A	—[g]
OS09	M	—[a]	Japan	—[a]	—[a]	Sexual	—[a]	—[a]	CRF01 AE	CRF01 AE	CRF01 AE	CRF01 AE	—[g]
OS10	F	—[a]	Kenya	—[a]	—[a]	Blood transfusion	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]
NG01	F	41	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG02	F	38	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	—[f]	—[f]	—[f]	—[g]
NG03	F	21	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	B	B	—[f]	D
NG04	M	14	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG05	F	36	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG06	F	26	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	CRF02 AG	CRF01 AE	CRF01 AE	A	—[g]
NG07	F	20	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG08	F	61	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	CRF02 AG	—[f]	—[f]	—[g]	—[g]
NG09	F	34	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	A	CRF01 AE	CRF01 AE	A	—[g]
NG10	F	35	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	C	CRF01 AE	C	—[g]	—[g]
NG11	M	33	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	B	B	—[g]	D
NG12	M	40	Africa	—[a]	—[a]	Sexual	—[a]	Sep/2002	D	—[f]	—[f]	—[g]	—[f]
JP02	M	31	Japan	1994	AC	Sexual	+	Jul/1999	B	—[f]	—[f]	—[g]	—[f]
KA14	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]
KA18	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	F	—[f]	—[f]	—[g]	—[g]
KA43	—[a]	—[a]	Africa	—[a]	—[a]	—[a]	—[a]	—[a]	G	CRF01 AE	CRF01 AE	G	—[g]

[a] No data.
[b] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, and E-SPEC for the second PCR.
[c] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers C-SPEC and F-SPEC for the second round PCR.
[d] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G for the second round PCR.
[e] Using primer BECO5 and BECO3 for the first round PCR and BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers 5′D and 3′D for the second round PCR.
[f] No product generated.
[g] Not done.

TABLE II. Sequence of Primers and Positions

Name	Sequence (5′–3′)	HXB2 no.[a]
J5′-2KSI	ATAAGCTTGCAGTGTAGCAGAAGAAGA	7003–7029
5′C2V3	TGTACACATGGAATTAGGCCAG	6963–6984
3′V3	ATGAATTCATTACAGTAGAAAAATTCCC	7363–7391
3′C2V3	ATTTCTGGGTCCCCTCCTGAGG	7313–7334
BECO5	GGCATCAAACAGCTCCAGGCAAG	7938–7960
BECO3	AGCAAAGCCCTTTCTAAGCCCTGTCT	8766–8791
BE-ANCH	TCCTGGCTGTGGAAAGATACCTA	7963–7985
B-SPEC	GTCCCCTCGGGGCTGGGAGG	8384–8403
E-SPEC	GTCTCAGTCCCTTGAGACTGCTG	8585[b]
F-SPEC	AACAGCTCTACCAGCTCTTTGCAAA	8720–8744
C-SPEC	AGACCCCAATACTGCACAAGACTT	8615–8638
5′E	CAGGAAAGGAATGAAAAGGATTTGTTA	8181–8207
3′E	ATAACCCTATCTGTCCACCCC	8693–8713
5′A	GANAACATGACCTGGCTGC	8094–8112
3′A	TCTATAACCCTATCTGTCCAGCCA	8693–8716
5′G	ACAATTACACATACCACATATACAGCC	8131–8757
3′G	TCTATAACCCTATCTGTCCAGTT	8694–8716
5′D	ACCACTAATGTGCCCTGGAACT	8037–8058
3′D	AGGAGGGTCTGAAATGACAGA	8356–8386
b-F	AGAGATGGCCACGGCTGCTT	
b-R	ATTTGCGGTGGACGATGGAG	

[a]HXB2 no. indicates primer position corresponding to nucleotide number of HXB2.
[b]HXB2 does not have sequence corresponding to primer, because of gap.

PCR of the v3 Region of the *gp120* Gene

The v3 region of HIV-1 provirus was amplified by nested PCR using primers 5′C2V3 and 3′V3 for the first round PCR reaction, and J5′-2KSI and 3′C2V3 for the second round reaction as described previously [Yagyu et al., 2002] (Table II). The PCR products were then subjected to 1% agarose gel electrophoresis at 100 V for 30 min, recovered from the gel, and used as a template for nucleotide sequencing.

Nucleotide Sequencing and Analysis

The sequencing reaction was carried out with a dideoxynucleotide cycle sequencing kit (Perkin Elmer, Wellesley, MA) using J5′-2KSI and 3′C2V3 as sense

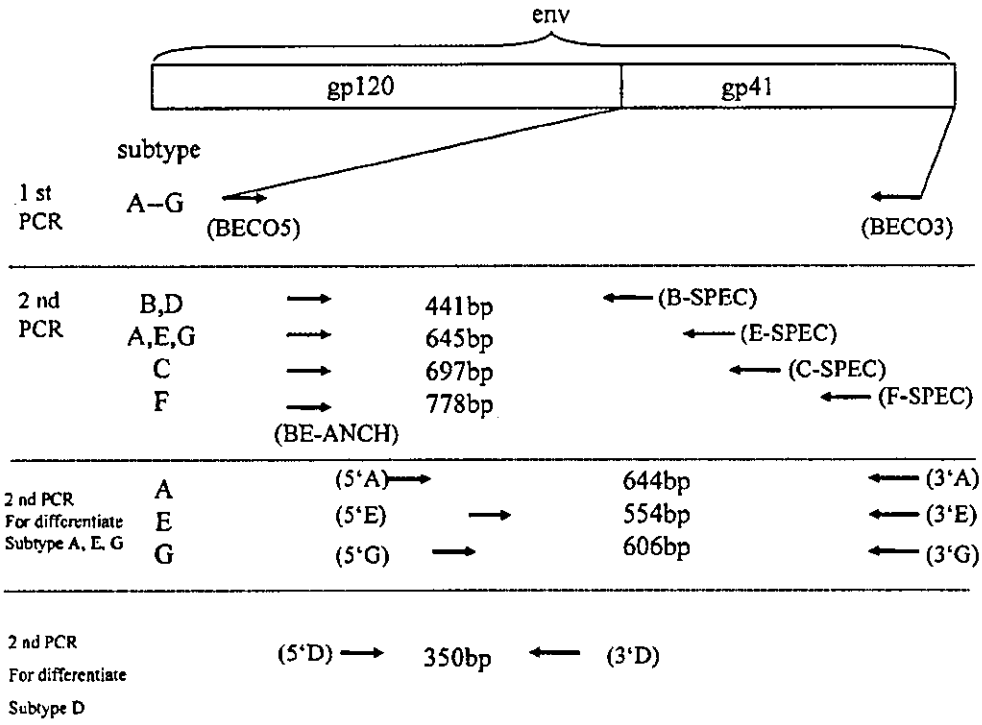

Fig. 1. Primer location and length of polymerase chain reaction (PCR) products.

and anti-sense primers for v3 region, respectively (Table II). The reaction products were analyzed by an automated DNA sequencer (ABI). The sequences of samples were aligned with references strains of subtypes A, B, C, D, F, G, CRF01_AE, and CRF02_AG, and SIV for analysis of v3 region by Clustal X [Thompson et al., 1997]. The subtypes were determined by phylogenetic trees, which were constructed using the neighbor-joining (NJ) method [Saitou and Nei, 1987].

RESULTS

Differentiation of Subtypes B and E by PCR With Subtype-Specific Primers

PCR to differentiate subtypes B and CRF01_AE was performed using samples from 33 subjects. A 441-bp PCR product, which was amplified between BE-ANCH and B-SPEC, was generated from the samples of 10 subjects, and a 645-bp PCR product, which was amplified between BE-ANCH and E-SPEC, was generated from the samples of 17 subjects. However, six subjects were negative, no PCR product was generated (Table I). All samples were positive for cellular beta-actin. This experiment was carried out to determine whether or not the primers B-SPEC and E-SPEC would react with the different subtypes. This information was considered useful for designing of new primers.

Determination of HIV-1 Subtype by Nucleotide Sequence Analysis of the v3 Region

In order to differentiate correct subtypes of the subjects, sequence analysis of the v3 region was performed, since this is considered the most reliable method. PCR of the v3 region of all samples generated successfully products of approximately 350 bp, which were then directly sequenced. The phylogenetic tree showed that the number of subjects of subtypes A, B, C, D, F, G, CRF01_AE, and CRF02_AG were 4, 6, 3, 6, 2, 3, 6, and 3, respectively (Tables I and III).

Designing of Env-Specific Primers for Differentiating Subtypes A, B, C, D, F, G, and CRF01_AE

For the first round PCR, the subtype-common primers, BECO5 and BECO3, were designed to differentiate subtypes B and CRF01_AE. Between BECO5 and BECO3, the subtype-unique conserved sequences of subtypes C and F were found, and used to design subtype C- and F-specific primers, respectively. However, the unique sequences specific for subtypes A, D, G, and CRF01_AE that could be used as anti-sense primers were not found. The results showed that the samples which were positive by PCR using BE-ANCH and subtype E-specific primers could be either subtype A, E, or G according to the sequence alignment, and this observation was in good agreement with a previous study [Yagyu et al., 2002]. Therefore, when the sample was positive for subtype A, E, or G by using the subtype E-specific primer, it was necessary to perform the second round PCR by using the first PCR product as the template with the alternative sets of subtype-specific primers, 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G (Table II, Fig. 2). Similarly, the sample which was positive for subtype B or D by using the subtype B-specific primer should be differentiated between subtypes B and D in the second round PCR using the first round PCR product as the template with the alternative set of primers 5′D and 3′D (Table II, Fig. 2).

Determination of Subtypes With Subtype-Specific Primers

The novel subtype-specific primers designed in this study were verified for specificity by testing with clinical specimens of the known subtypes. The primers used for the first round PCR were BECO5 and BECO3, and then the second round PCR was performed using the first round PCR product as the template, BE-ANCH as the 5′ primer and B-SPEC, C-SPEC, E-SPEC, and F-SPEC as the 3′ primers (Table II, Figs. 1, 2, and 3A). The primers C-SPEC and F-SPEC generated products of 675 and 778 bp, and the samples were identified as subtype C and F, respectively. A PCR product of 440 bp was also generated, possibly due to the annealing of the B-SPEC primer to the proviral DNA of subtypes B and D in the sample. Then, using the first PCR product as template, the second PCR was performed with 5′D and 3′D primers for detecting subtype D, and a 350-bp product was generated (Figs. 1, 2, and 3B). Similarly, E-SPEC annealed to subtypes A, E, and G, and generated a 650-bp product. Then, using the first PCR product as template, the second PCR was performed with a mixture of the primers 5′A, 3′A, 5′E, 3′E, 5′G, and 3′G, and

TABLE III. Subtypes Determined by v3 Sequence and PCR Using Novel Primers

Subtype by v3 sequence	No. of samples with subtype by PCR using novel primers								
	A	B	C	D	F	G	CRF01_AE	ND	Total (n = 33)
A	3							1	4
B		5						1	6
C			3						3
D				5				1	6
F					2				2
G						3			3
CRF01_AE							6		6
CRF02_AG	2							1	3

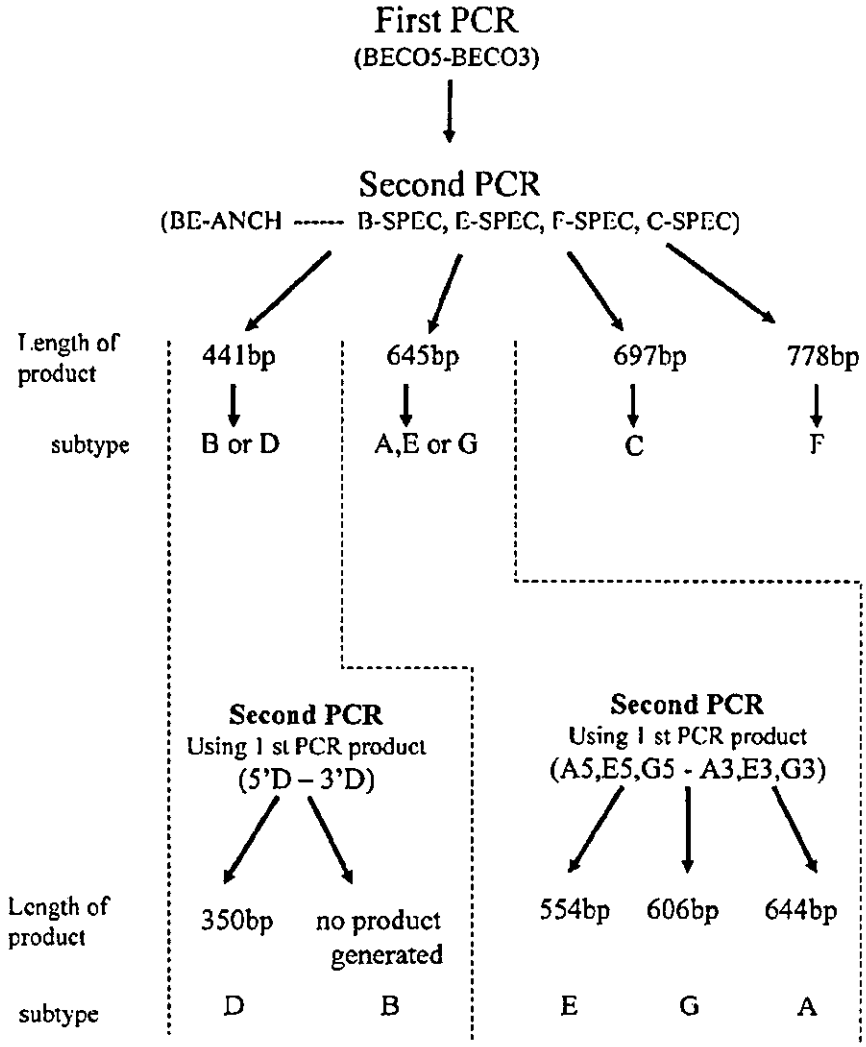

Fig. 2. Procedure of PCR for differentiation of subtypes with newly designed primers.

products of 644, 554, and 606 bp were generated for subtypes A, E, and G, respectively (Figs. 1, 2, and 3C). A total of 33 subjects were tested by this procedure using the newly designed primers. When BE-ANCH was used as a 5′ end primer in combination with B-SPEC, E-SPEC, C-SPEC, and F-SPEC as 3′ end primers, and the first round product was used as the template, PCR products of 441, 645, 697, and 778 bp were generated from 10, 14, 3, and 2 out of the 33 subjects, respectively (Tables I and III). In addition, when the alternative second round PCR for differentiation of subtypes A, G, and CRF01_AE was performed using the first round PCR product as a template and a mixture of the 5′A and 3′A, 5′E and 3′E, 5′B and 3′G primer pairs, 644 bp (subtype A), 554 bp (CRF01_AE), and 606 bp (subtype G) products were generated from 5, 6, and 2, out of the 13 subjects, respectively. Furthermore, when 5′D and 3′D were used in the alternative second round PCR to differentiate subtype D from subtype B, a 350 bp (subtype D) product was generated from 6 out of 12 subjects. The other 6 of the 12 subjects were therefore identified as subtype B.

DISCUSSION

The PCR for differentiation of subtype B and CRF01_AE was carried out as described previously [Yagyu et al., 2002]. The primers B-SPEC and E-SPEC have cross-reaction with other subtypes, except for subtype B and CRF01_AE. The B-SPEC primer anneals subtypes B and D, and the E-SPEC primer anneals subtypes A, C, G, and CRF01_AE. The result of PCR of cellular beta-actin was positive for all samples, suggesting that the DNA in samples was intact by the time of testing and the extraction procedure was successful. The samples that did not generate any products were considered not to have been amplified by the B-SPEC and E-SPEC primers, possibly because there was an insufficient copy number of the HIV-1 provirus or because it was subtype F [Yagyu et al., 2002].

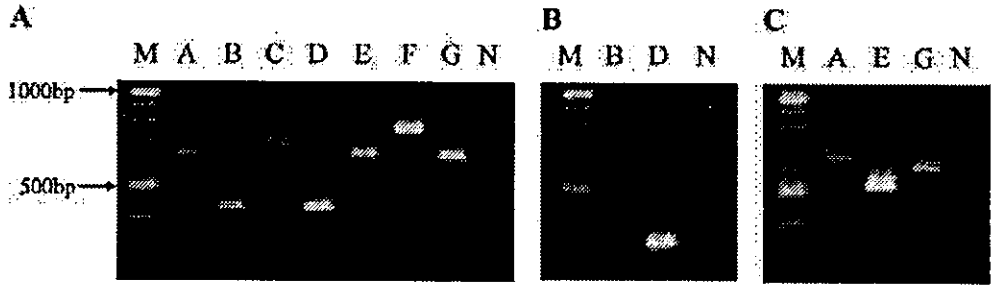

Fig. 3. Determination of subtypes with subtype-specific primers. A: PCR with primers (BECO5 and BECO3 for the first round PCR and, BE-ANCH, B-SPEC, E-SPEC, and newly designed subtype-specific primers C-SPEC and F-SPEC for the second round PCR) of subtype A (lane A), B (lane B), C (lane C), D (lane D), CRF01_AE (lane E), F (lane F), and G (lane F). PCR products were subjected to agarose gel electphoresis with 100 bp ladder markers (lane M) and negative control (lane N). B: PCR with primers (BECO5 and BECO3 for the first round PCR and, 5'D and 3'D for the second round PCR) of subtype B (lane B) and D (lane D). PCR products were subjected to agarose gel electrophoresis with 100 bp ladder markers (lane M) and negative control (lane N). C: PCR with primers (BECO5 and BECO3 for the first round PCR and, 5'A, 3'A, 5'E, 3'E, 5'G, and 3'G for the second round PCR) of subtype A (lane A), CRF01_AE (lane E), and G (lane G). PCR products were subjected to agarose gel electphoresis with 100 bp ladder markers (lane M) and negative control (lane N).

Among the patients (BRON04, NG02, NG08, NG12, JP02, and KA18) who were negative for PCR for differentiation of subtypes B and E, BRON04 and KA18 were subtype F as expected. And, the subjects, NG02, NG08, NG12, and JP02, were subtypes A, CRF02_AG, D, and B, respectively.

The newly designed subtype-specific primers could be used as anti-sense primers against BE-ANCH, and could generate PCR products of different lengths. Specific primers for subtypes C and F which act as the anti-sense primers against BE-ANCH were also designed. However, it was rather difficult to design a subtype-specific for A, B, D, G, or CRF01_AE, because subtypes A, E, and G and subtypes B and D, respectively, were similar to each other. After the second PCR with primers BE-ANCH, B-SPEC, C-SPEC, E-SPEC, and F-SPEC, the sample which was positive for the subtype B-specific primer was further differentiated between subtypes B and D by using the subtype D-specific primers 5'D and 3'D. By this strategy, the primers were able to recognize only subtype D, since it was impossible to design a subtype B-specific primer for the second PCR. Since the subtypes B and D are very closely related, an attempt was made to differentiate the subsubtypes by PCR. The results showed that PCR was able to separate them incompletely in this experiment. However, because subtypes B and D are predominant in different countries, the differentiated subtypes B and D could provide important information on the course of an HIV strain newly imported to a particular country.

Three samples (OS08, NG06, NG08) of CRF02_AG determined by nucleotide sequence were differentiated as subtype A by PCR with the novel primers. The envelope gene of CRF02_AG consists of subtype A and G. Therefore, it may be impossible to design specific primers for CRF02_AG, when only the genome of gp41 is used for subtype determination.

The subtypes of all subjects determined by PCR using newly designed primers were in complete agreement with those determined by nucleotide sequence analysis of the v3 region except for CRF02_AG. Nevertheless, there were four patients, who did not generate any positive signals. This indicates that the relatively lower sensitivity of PCR with novel primers might not be due to the low copy number of proviral DNA; rather, it might be due to mismatched of the primer, since point mutations readily occur in the HIV-1 genome.

The newly designed primers functioned accurately and conclusively. In comparison with PCR as a method for the determination of subtypes, sequence analysis requires better-trained personnel, more expensive reagents, and more equipment and time. The PCR method is useful for developing countries in which the burden of HIV/AIDS has increased dramatically. However, although the PCR method has the above advantages, it also has a shortcoming in that it differentiates subtypes only in the gp41 region. In order to evaluate this method accurately, a larger number of samples should be tested.

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