ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2005) 23: 143-152

HIV-1 Subtyping Using *gag*/*env* Heteroduplex Mobility Assay and Peptide Enzyme-Linked Immunosorbent Assay

Wantanee Simparak[1], Uraiwan Kositanont[1], Ruengpung Sutthent[1], Punneeporn Wasinrapee[2], Thanyanan Chaowanachan[2] and Chantapong Wasi[1]

SUMMARY Two HIV-1 subtypes have accounted for virtually all infections in Thailand: subtype B', found mainly in injection drug users (IDUs), and CRF01_AE (initially subtype E), found in over 90% of sexually infected persons and increasingly in IDUs in recent years. During 1997-1998, 227 blood samples were collected from HIV-1 infected individuals consisting of 92 mothers, 35 children and 100 IDUs. The blood samples were subtyped by heteroduplex mobility assay (HMA) and peptide enzyme-linked immunosorbent assay (PEIA). Using *gag* and *env* HMA, CRF01_AE and subtype B' accounted for 96-97% and 3-4% of both the mothers and the children, respectively. In the IDU group, 10% of the plasma samples could only be performed by *gag* HMA and gave the result as CRF01_AE. CRF01_AE and subtype B' using PEIA accounted for 67% and 33% of the IDUs. There was 100% concordance of the results between *gag* HMA and *env* HMA. Ninety-five percentages of concordant results were observed between HMA and PEIA. Of the 6/134 (5%) subjects with discordant results, nucleotide sequencing, used as a gold standard, confirmed the HMA result. In this study, HIV-1 was successfully genotyped by HMA and PEIA. However, a comparison of the subtyping results between HMA and PEIA revealed that HMA was slightly more accurate than PEIA.

The human immunodeficiency virus (HIV) is grouped into two types: HIV-1, the predominant HIV type throughout the world, and HIV-2, which is less widespread and still primarily found in West Africa. HIV-1 is further classified into three groups: group M (major) which is globally prevalent, group O (outlier), and group N (non-M/non-O).[1] HIV-1 groups and subtypes are unevenly distributed in the world. Over the years the definition of group M subtypes was adapted in the light of emerging recombinants. Representatives of different "pure" (non-recombinant) subtypes A, B, C, D, F, G, H, and J and circulating recombinant forms (CRFs) were proposed based on near-full-length genome analysis, as determined by the HIV Sequence Database.[2,3]

In Thailand, the HIV-1 epidemic started abruptly in 1988 with the introduction of subtype B' and CRF01_AE,[4] initially called subtype E (a descendant of a hybrid HIV-1, generated by a recombination of subtypes A and E). In the early epidemic, these two strains appeared independently in distinct high-risk populations: subtype B' among injecting drug users (IDUs) and CRF01_AE among those who were heterosexually exposed.[5] HIV-1 subtype B' is

From the [1]Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand, [2]Thailand MOPH-US CDC Collaboration, A joint activity of the Thailand Ministry of Public Health & the U.S. Centers for Disease Control and Prevention.
Correspondence: Uraiwan Kositanont
E-mail: gruks@mahidol.ac.th

still common among infected IDUs in Bangkok; however, CRF01_AE is found with increasing proportion among young IDUs.[6] Dual infection with HIV-1 subtypes B' and CRF01_AE was observed by Artenstein *et al*. in 1994,[7] which provided an opportunity for recombination between these two subtypes in the Thailand epidemic. In Thailand, the CRF01_AE/B recombinant strains were identified in both heterosexual and IDU exposed individuals[8,9] and additionally a CRF01_AE/subtype C recombinant has been identified in Chonburi Province.[10]

The development of a reliable and economical screening technique to track prevalent HIV-1 subtypes and emerging recombinant isolates worldwide is important for developing a protective vaccine. Although full-length sequencing remains the most accurate technique to characterize viral genomes, it is expensive for use in large-scale studies, especially for the developing nations that are mostly affected by the HIV epidemics. The heteroduplex mobility assay (HMA) is based on an analysis of selected regions in the *gag* gene (*gag* HMA) and *env* gene (*env* HMA).[3,11,12] Moreover, the peptide enzyme-linked immunosorbent assay (PEIA) using peptides corresponding to the third hypervariable (V3) region of HIV-1 gp120 allows for rapid subtyping of HIV-1 in field conditions.[6,13-16]

Both techniques are less time-consuming and labor intensive than genetic sequencing; therefore these two assays are valuable for screening viral subtypes in a field setting. The objectives of this study were to determine HIV-1 subtypes in infected Thai subjects by genetic and serologic techniques and to compare the results of *gag*/*env* HMA and PEIA.

MATERIALS AND METHODS

Subjects and clinical samples

Blood samples were obtained from 92 HIV-1 seropositive mothers and 35 HIV-1 infected children, who attended the Pediatric Clinic in Siriraj Hospital and Charoenkrung-Pracharak Hospital during 1997-1998. The 92 mothers were divided into two groups, 42 transmitting mothers (TM) who were the mothers of infected children and 50 non-transmitting mothers (NTM) who were the mothers of uninfected children. Each mother and infant (C) pair had the same recorded code number. The blood samples of the mothers and children were collected on the first visit (1-3 months) after delivery. After the first visit, the infants were followed until 18 months old. All mothers were asymptomatic at the time of sample collection. No antiretroviral therapy was given to mothers or their babies after delivery.

Plasma samples were obtained from 100 HIV-1-seropositive injecting drug users (IDUs) in 1998 from the National HIV Repository and Bioinformatic Center (NHBRC), Thailand, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University.

The study protocol was approved by the Ethical Review Board of the Research Committee, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

Sample preparation for polymerase chain reaction (PCR)

Peripheral blood mononuclear cells (PBMCs) were isolated from blood samples using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The cells were lysed according to the protocol as previously described.[17] The cell lysates were stored at -20^{o}C until tested. For plasma samples, total RNA was extracted from 140 µl of the lower part of the pellet of 420 µl of plasma centrifuged at 4^{o}C, 20,000 x *g* for 70 minutes by QIAamp® Viral RNA Mini Kit (Qiagen, Hilden Germany) according to the instruction of the manufacturer. The extracted RNA was stored in 60 µl of AVE buffer (RNase-free water and 0.04% sodium azide) at -70^{o}C until used.

Polymerase chain reaction (PCR)

gag *DNA PCR*

HIV-1 DNA was amplified as previously described.[3] H1G777 (5'-TCACCTAGAACTTTGAATGCATGGG) sense and H1P202 (5'-CTAATACTGTATCATCTGCTGCTCCTGT) antisense primers were used as first round primers. For nested PCR, H1Gag1584 (5'-AAAGATGGATAATCCTGGG) sense and g17 (5'-TCCACATTTCCAACAGCCCTTTTT) antisense primers amplified a 460-bp *gag*

gene fragment corresponding to the region coding for amino acid 132 of p24 to amino acid 40 of p7. The amplification conditions were as follows: 1 cycle at 94°C for 2 minutes, followed by 35 cycles at 94°C for 30 seconds, at 50°C for 30 seconds, and at 72°C for 60 seconds, and a final extension at 72°C for 7 minutes.

env *DNA PCR*

HIV-1 DNA was amplified as previously described.[12] The outer primers were ED5 (5'-ATGGGATCAAAGCCTAAAGCCATGTG) and ED12 (5'-AGTGCTTCCTGCTGCTCCCAAGAACCCAAG). The amplification conditions were as follows: 5 cycles at 94°C for 1 minute, at 55°C for 1 minute and at 72°C for 90 seconds followed by 35 cycles at 94°C for 1 minute, at 55°C for 45 seconds, at 72°C for 90 seconds, and a final extension at 94°C for 1 minute, at 55°C for 45 seconds and at 72°C for 10 minutes. For nested PCR, the inner primers were ED31 (5'-CCTCAGCCATTACACAGGCCTGTCCAAAG) and ED33 (5'-TTACAGTAGAAAAATTCCCCTC) to enable amplification of 500-bp of C2-C3 from the gp 120 region. The amplification conditions were 1 cycle at 94°C for 1 minute and at 55°C for 1 minute followed by 35 cycles of the same cycling conditions as mentioned above.

Heteroduplex mobility assay (HMA)

HIV-1 subtype was determined by HMA as described elsewhere.[3,12] Heteroduplexes were obtained by mixing 5 µl of the amplified fragments of the unknown subtype sample with the same amount of each of the amplified reference plasmids in *gag*-HMA (Table 1) and *env* HMA (Table 2), kindly provided by the NIH AIDS Research and Reference Reagent Program, National Institutes of Health, in 1x annealing buffer (1 M NaCl, 100 mM Tris-HCl, 20 mM EDTA); the mix was then denatured by heating for 2 minutes at 94°C and rapidly cooled in wet ice. Heteroduplexes and homoduplexes were separated by electrophoresis on a 5% polyacrylamide gel (29:1 acrylamide:bis-acrylamide ratio), with the addition of 20% urea for gag HMA only, at 250 Volts for 2 hours and 30 minutes. The gels were then stained with ethidium bromide and visualized under

Table 1 *gag* HMA reference panel[3]

Reference abbreviation	Reference plasmids	Origin	Contributors*	Cloned fragment**	Plasmid vector
A1	pVI310 (A1)	Rwanda	ITM/HMJF	HG00-HG01	pBluescript
A2	pCI4 (A2)	Cote d'Ivoire	ITM/HMJF	HG00-HG01	pBluescript
A3	pVI57 (A3)	DRC	ITM/HMJF	HG00-HG01	pBluescript
A4	pK29 (A4)	Kenya	ITM/HMJF	HG00-HG01	pBluescript
B1	pTB132(B1)	Thailand	ITM/HMJF	HG00-HG01	pBluescript
B2	pUG280 (B2)	Uganda	ITM/HMJF	HG00-HG01	pCRII-TOPO
B3	pPIC63 (B3)	Belgium	ITM	HGHMA101-HGHMA1317	pCRII-TOPO
B4	pPIC335 (B4)	Belgium	ITM	HGHMA101-HGHMA1317	pCRII-TOPO
C1	pDJ259 (C1)	Djibouti	ITM/HMJF	HG00-HG01	pCRII-TOPO
C2	pVI313 (C2)	DRC	ITM/HMJF	HG00-HG01	pBluescript
C3	pUG268 (C3)	Uganda	ITM/HMJF	HG00-HG01	pCRII-TOPO
C4	pZM145 (C4)	Zambia	ITM	HGHMA101-HGHMA1317	pCRII-TOPO
C5	pZM192 (C5)	Zambia	ITM	HGHMA101-HGHMA1317	pCRII-TOPO
E1	pTN238 (AEcm240 1)	Thailand	ITM/HMJF	HG00-HG01	pCRII-TOPO
E2	pCA10 (AEcm240 2)	Cameroon	ITM	HGHMA101-HGHMA1317	pCRII-TOPO
E3	pVI2064 (AEcm240 3)	Thailand	ITM	HGHMA101-HGHMA1317	pCRII-TOPO

*ITM, Institute of Tropical Medicine, Antwerp, Belgium; HMJF: Henry M. Jackson Foundation, Rockville, Maryland, USA.
**Primers used for amplification of the cloned *gag* fragments.

Table 2 *env* HMA reference panel[12]

Subtype	HIV-1 strain	Accession number	Cloned fragment	Plasmid vector	Size (bp)	*Eco* RI sites	*Hind* III sites	Drug	Contributor
A2	IC144	n/a	ED5/ED12	pCR1	4381	1	2	Kan	A
A3	SF170	M66533	gp160	pCR2	6550	2	1	Kan/Amp	B
B1	BR20	U08797	gp160	pCR2	6772	2	1	Kan/Amp	C
B2	TH14	U08801	gp160	pCR2	6750	2	2	Kan/Amp	C
B3	SF162	M65024	6.6kb 3'end	pUC19	3952			Amp	B
C1	MA959	U08453	gp160ED5/E	pCR2	6783	2	1	Kan/Amp	D
C2	ZM18	L22954	D12	pCR1	5466	2	2	Kan	A
C3	IN868	U07103	ED5/ED12	pCR1	4150	1	1	Kan	E
E1	TH22	U09131	gp160	pCR2	6778	2	2	Kan/Amp	C
E2	TH06	U08810	gp160	pCR2	6294	2	2	Kan/Amp	C
E3	CAR7	n/a	ED5/ED12	pCR2	5197	2	1	Kan/Amp	F

a UV transilluminator. Those heteroduplexes formed by the sample and the reference DNA that migrate faster (close to the homoduplexes at the bottom of the gel) are supposed to be most closely related to the specific reference DNA.

Peptide enzyme-linked immunosorbent assay (PEIA)

The screening PEIA technique with a minor modification was described in detail elsewhere.[13] Briefly, a panel of gp120 V3 synthetic peptides based on the 14 amino acids at the tip of the V3-loop (the principle neutralizing domain) were used to construct the E and B' peptides. A 15th non-viral amino acid (aspartic acid) was added to the N-terminus to increase the overall negative charge and thus enhance peptide-binding to the plate. The amino acids of the 4 peptides were PEP-E (D*TSITIGPGQVFYRT), PEP-B' (D*KSIHLGPGQAWYTT), PEP-BR (D*KSIHLGPGRAWYTT) and PEP-MN (D*KRIHLGPGRAFYTT). These peptides were synthesized for the HIV-1 subtypes E and B' and were supplied by the Centers for Disease Control and Prevention (CDC) in Atlanta, GA. Each synthetic peptide was then used to coat microtiter plates, which were then used to test HIV-positive sera by indirect enzyme immunoassay. A positive result was defined by an OD above the cutoff (OD of negative samples plus 5 SD). Specimens were classified as either subtype B or subtype E antibody positive when the OD for one V3 peptide was more than two-fold the OD of the other. Sera demonstrating reactivity to both peptides, but less than a two-fold differential, were classified as dual subtype (B/E). Samples were classified as non-typeable if the OD was less than the cutoff OD.

DNA sequencing and phylogenetic analysis

Samples with discordant results of HMA and PEIA were confirmed by nucleotide sequencing which is regarded as the "gold standard" method for HIV subtyping. For the *gag* gene, a 460-bp fragment from *gag* DNA PCR was amplified with the previously described primer pairs of H1Gag1584 and g17. For the *env* gene, a 530-bp fragment corresponding to the *env* C2-V4 region of gp120 was amplified with the primer pairs JH33 (5'-CTGTTIAATGGCAGICTAGC) and JH48 (5'-RATGGGAGGRGYATACAT). Double-stranded PCR fragments were sequenced in both directions using an ABI PRISM fluorescent dye-labeled determinator sequencing kit (ABI-PRISM Dye Terminator Cycler Sequencing Ready Reaction Kit, Perkin Elmer Cetus, Norwalk, CT, USA). DNA were generated using an automated sequencer (ABI Model 370, Perkin Elmer Cetus).

Analysis of data

The HIV-1 subtypes obtained by *gag*/*env* HMA were compared to the nucleotide sequences of the reference strains representing the different genetic subtypes in the *gag* and *env* genes. DNA sequences were aligned with complete genome reference sequences of accession numbers (subtypes) as follows: X52154 (SIV), AJ006022 (HIV-1 group N), L20517 (HIV-1 group O), L02571 (HIV-1 group O), U51190 (A), AF004885 (A), M17451 (B), M17449 (B), U52953 (C), AF110967 (C), K03454 (D), M27323 (D), U51189 (E), U54771 (E)AF005494 (F), AF077336 (F), AF061642 (G), AF084936 (G), AF190128 (H), AF005496 (H), AF082395 (J) and AF082394 (J) with CLUSTAL and Bioedit software.

RESULTS

Detection of PCR products before HMA performance

For *gag* PCR amplification, the amplification products of 460 bp detected in all samples of the TM, NTM and C groups were further used in *gag* HMA. HIV-1 RNA in the plasma samples in the IDU group failed to be amplified by RT-PCR. For more concentrated RNA, the pellet of 420 µl plasma centrifuged at 4°C, 13,000 rpm for 70 minutes was further extracted. Ten out of 100 (10%) plasma samples gave positive results by nested PCR.

For *env* PCR amplification, the amplification products of 500 bp were detected in 37/42 (88%), 45/50 (90%) and 29/35 (83%) samples in the TM, NTM and C groups, respectively. HIV-1 RNA in all concentrated plasma samples in the IDU group failed to be amplified by RT-PCR.

Determination of HIV-1 subtypes by HMA

Figs. 1 and 2 show the typical patterns of *gag* and *env* HMA CRF01_AE, respectively. In each lane, three types of bands are visible: (i) the double-stranded homoduplex bands at the bottom of the gel; (ii) the single-stranded band in the middle of the gel, resulting from the fraction of DNA molecules which have not reannealed during the reaction process; and (iii) the heteroduplex bands at different positions in the gel lanes. The HIV-1 subtype was determined by the fastest migrating heteroduplex bands, which were closely related to the homoduplex bands of the specific reference subtype.

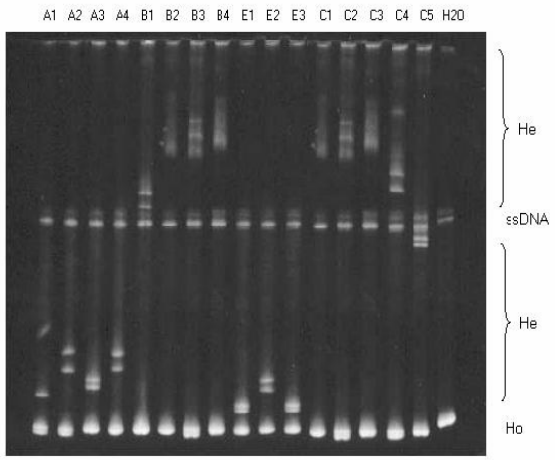

Fig. 1 *gag* HMA of one sample from an infected individual showing HIV-1 subtype E (CRF01_AE) on a 5% polyacrylamide gel containing 20% urea. Note: He, Heteroduplex; Ho, Homoduplex; ssDNA, single-stranded DNA; and A1 to C5, the full set of the 16 subtype reference plasmids given in Table 1.

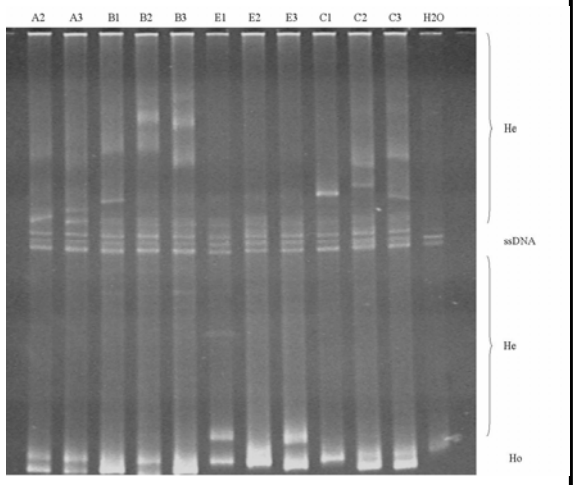

Fig. 2 *env* HMA of one sample from an infected individual showing HIV-1 subtype E (CRF01_AE) on a 5% polyacrylamide gel. Note: He, Heteroduplex; Ho, Homoduplex; ssDNA, single-stranded DNA; and A2 to C3, the full set of the 11 subtype reference plasmids given in Table 2.

HIV-1 subtyping by *gag* and *env* HMA

The genetic HIV-1 subtyping by HMA performed among the TM, NTM, C and IDU groups is shown in Table 3. In the TM group, 38/42 (90%) and 34/42 (81%) samples were successfully subtyped by *gag* and *env* HMA, respectively. Of 38 typeable samples by *gag* HMA, 37 (97%) and 1 (3%) samples were classified as CRF01_AE and subtype B, respectively. Of 34 typeable samples by *env* HMA, 33 (97%) and 1 (3%) samples were classified as CRF01_AE and subtype B, respectively.

In the NTM group, 50/50 (100%) and 42/50 (84%) samples were successfully subtyped by *gag* and *env* HMA, respectively. Of 50 typeable samples by *gag* HMA, 48 (96%) and 2 (4%) samples were classified as CRF01_AE and subtype B, respectively. Of 42 typeable samples by *env* HMA, 40 (95%) and 2 (5%) samples were classified as CRF01_AE and subtype B, respectively.

In the C group, 28/35 (80%) and 25/35 (71%) samples were successfully subtyped by *gag* and *env* HMA, respectively. Of 28 typeable samples by *gag* HMA, 27 (96%) and 1 (4%) samples were classified as CRF01_AE and subtype B, respectively. Of 25 typeable samples by *env* HMA, 24 (96%) and 1 (4%) samples were classified as CRF01_AE and subtype B, respectively.

In the IDU group, 10/100 (10%) samples were subtyped as CRF01_AE by *gag* HMA while none as subtyped by *env* HMA.

HIV-1 subtyping using peptide enzyme-linked immunosorbent assay (PEIA)

Antibodies to the V3 loop of HIV-1 in 38/39 (97%) plasma samples of the TM group, in 41/45 (91%) samples of the NTM group and in 46/50 (92%) samples of the IDU group were interpreted as monoreactive to either peptide E or peptide B'. Nine of 134 (8%) plasma samples from 1, 4 and 4 subjects in the TM, NTM and IDU groups, respectively, were classified as non-typeable.

The results of the PEIA in the TM, NTM and IDU groups are shown in Table 3. All 38 (100%) samples from the TM group were classified as CRF01_AE. In the NTM group, 38/41 (93%) and 3/41 (7%) cases were classified as CRF01_AE and subtype B, respectively. In the IDU group, 31/46 (67%) samples and 15/46 (33%) samples were classified as CRF01_AE and subtype B (Thai B with GPGQ V-3 loop crown) whereas one sample reacted to peptide BR (Thai B with GPGR V-3 loop crown), respectively.

Concordant and discordant results between *gag* and *env* HMA and PEIA

There was 100% concordance between the results of *gag* HMA and *env* HMA for HIV-1 subtyping in the TM, NTM and C groups. It was found that there was 95% concordance between the results of *gag*/*env* HMA and PEIA. There were 6 (5%) samples with discordant results between HMA and PEIA (Table 4).

Table 3 HIV-1 subtypes of transmitting mother (TM), non-transmitting mother (NTM), Infant (C) and injecting drug user (IDU) groups by *gag*/*env* HMA and PEIA

Group	*gag* HMA		*env* HMA		PEIA	
	Subtype B	CRF01_AE	Subtype B	CRF01_AE	Subtype B	CRF01_AE
TM	37 (97%)	1 (3%)	33 (97%)	1 (3%)	37 (97%)	1 (3%)
NTM	48 (96%)	2 (4%)	40 (95%)	2 (5%)	38 (93%)	3 (7%)
C	27 (96%)	1 (4%)	24 (96%)	1 (4%)	ND	ND
IDU	10 (100%)	0 (0%)	ND	ND	31 (67%)	15 (33%)

ND, not done

Table 4 Discordant results of heteroduplex mobility assay (HMA) and V3-peptide enzyme-linked immunosorbent assay (PEIA) of discrepant HIV-1 subtypes using nucleotide sequencing as "gold standard"

Code No.	Group	HIV-1 Subtyping by				
		HMA		PEIA	Sequencing	
		gag	*env*		*gag*	*env*
MC177	NTM	E	E	B	E	NT
MC201	NTM	B	B	E	B	NT
MC211	NTM	E	E	B	E	NT
MC216	NTM	E	E	B	E	E
M346-02	TM	B	B	E	B	NT
TK109	IDU	E	E	B	E	NT

Note: NT, Non-typeable; E, CRF01_AE; B, subtype B

Discordant results between HMA and PEIA confirmed by nucleotide sequencing

The six samples with discordant results between HMA and PEIA were further tested by nucleotide sequencing. The comparison of the HMA and PEIA with DNA sequencing is shown in Table 4. The nucleotide sequencing of all 6 samples, with either subtypes B or E, agreed with the HMA. The data of this study also showed that *gag* nucleotide sequencing was more reliable for HIV-1 subtyping than *env* nucleotide sequencing. Of 6 samples, only one sample could be subtyped by *env* nucleotide sequencing.

DISCUSSION

Studies on the current circulating subtypes of HIV-1 in different parts of the world yield important information for epidemiological data and vaccine development. Different HIV-1 subtypes occur worldwide with different patterns of geographical distribution. Genetic subtyping using heteroduplex mobility assay (HMA)[3,11,12] and serological testing using synthetic peptides from consensus sequences of HIV-1 genomes, especially from the gp120 V3 loop,[6,13-16] have been used for HIV-1 subtyping.

In this study, it was found that the predominance of CRF01_AE with 96% in the heterosexual transmission group and 67% in the IDU group fully correlated with the viral pattern that was documented for these risk groups worldwide. The predominance of the HIV-1 CRF01_AE strain among the Thai heterosexual population during the time of this study was similar to other studies which reported that CRF01_AE accounted for more than 90% of the heterosexual transmission group.[18,19]

In the IDU group during this study, the data revealed 67% to be infected with CRF01_AE and 33% with subtype B. HIV-subtyping using PEIA in 1998 demonstrated that 78% to 79% and 21% to 22% were of CRF01_AE and B, respectively.[16, 20] The comparative data in the same period revealed that the proportion of HIV-1 subtypes E and B was slightly lower than that in the previously mentioned studies. This might have resulted from the difference in the HIV subtype distribution by age of infected IDUs. The proportion of HIV-1 subtypes E and B by age $\leq$ 20, 21-25, and 26-30 years old as 84.3% *vs* 15.7%, 77.3% *vs* 22.7%, and 69% *vs* 32.1%, respectively,[16] supported the results of the IDUs' mean age of 29.3 with 67% *vs* 33% identified as subtypes E and B, respectively, in this study.

Previous reports have suggested that HMA is an effective method for evaluating the genetic subtypes of HIV-1 in different geographic regions. However, the technical performance of the HMA may be influenced by the relative prevalence of the different subtypes in the target population. The HMA gives excellent results in populations in which a particular subtype prevails, such as HIV-1 subtype B in

Western European countries and the United States[21] and HIV-1 subtype E in Vietnamese drug users,[22] and even in populations which have divergent HIV-1 subtypes, such as CRF01_AE in Thailand[6] and CRF02_AG in Russia.[23]

The data in this study have confirmed that *gag* HMA is an effective HIV-1 subtyping method. 116/127 (91%) samples in the heterosexual transmission group were successfully subtyped by HMA whereas 10/100 (10%) samples in the IDU group were genotyped by *gag* HMA only. The unsuccessful subtyping in plasma samples collected from IDUs by PCR might have resulted from the plasma storage. It was found that there was 100% concordance between *gag* HMA and *env* HMA results regarding specificity; however, a comparison of subtyping results between *gag* HMA and *env* HMA revealed that *gag* HMA was slightly more sensitive than *env* HMA. *Gag* HMA could subtype a higher percentage of samples than *env* HMA, i.e. 91% *vs* 80%, 100% *vs* 86%, 80% *vs* 71%, and 10% *vs* 0% in the TM, NTM, infant and IDU groups, respectively. This could be contributed to the lower sequence diversity in the *gag* gene compared with the *env* gene.[12] Thus, the *gag* HMA could be used to increase detection levels in situations where the *env* HMA is insufficiently sensitive. The data in this study clearly demonstrated that *gag* HMA was more effective for HIV-1 subtyping than *env* HMA. However, both *gag* and *env* HMA can be used for HIV-1 subtyping in areas where CRFs are highly probable to occur.

Previous studies have suggested that serotyping by PEIA using envelope peptides provides a good tool determining the prevalence of different HIV-1 subtypes. Subtyping using PEIA in countries such as Thailand, where only two subtypes of HIV-1 were circulating, has been shown to be very effective for detecting antibodies to the V3 loop.[6,13-16] However, this technique has not been very successful in areas where several antigenically related subtypes of HIV-1 co-circulate, due to a high level of cross-reactivity.[25,26] Among 15/46 (33%) samples classified as subtype B, one sample reacted to peptide BR (Thai B with GPGR V-3 loop crown). The presence of the GPGR motif in the V3 loop of genotype B suggests that it is more closely related to North American and European strains.[27] Nine of 133 (8%) serum samples did not react to peptide, classified as non-typeable, because of insufficient levels of antibody.

All 6 samples with discordant results, identified by PEIA did not agree with *gag* nucleotide sequencing. The 5 unidentified samples by *env* sequencing might have resulted from mixing *env* regions of heterogeneous population variants. In 3 of 6 samples, the PBMC-derived viral *env* gene populations also showed a high degree of genetic relatedness as indicated by the formation of homoduplexes with a large amount of smearing.[28] The discordance between serological subtyping by PEIA and HMA/genetic sequencing was consistent with other previous studies.[21,29,30] The discordance in this study might have occurred when the amino acid sequence of the infecting virus closely resembled those of more than one consensus peptide antigen or when the observed V3 crown motif of the strain was atypical for the genetic subtype present. Van der Groen *et al*.[29] indicated that V3-loop PEIA methodologies used in different laboratories correlated poorly with genetic subtyping, and their accuracy in predicting HIV-1 subtypes varied considerably in sera of Belgian individuals infected with different HIV-1 subtypes. The poor correlation between serotyping and genetic subtyping was partly due to the simultaneous occurrence of subtype-specific octameric sequences at the tip of the V3-loop of viruses belonging to different genetic subtypes. Murphy *et al*.[15,30] reported that the accuracy of serotyping with the V3 peptide depends on the infected host's immune response to an antigen coded by a very small genetic domain which may significantly affect antigenicity. By contrast, the genetic subtyping methods used in this investigation are dependent on the analysis of a much larger domain, in this case over 10 times as large. Consequently, genotypic analysis can be expected to be more specific.

Although PEIA has a simplified performance, the accuracy is lower than HMA. The data in this study demonstrated that the discordant results could give rise to erroneous information in HIV-1 PEIA. In a case of an HMA-based failure to ascribe subtypes in the IDU group either because of PCR failure or insufficient PCR product, PEIA is helpful for subtyping. In this study, the CRF01_AE identified by *gag* HMA was detected in only 10 IDU samples whereas 67% for CRF01_AE and 33% for sub-

type B were subtyped by PEIA. Genetic recombination between HIV-1 subtypes might be expected to result in a further reduction of the specificities of serotyping assays and may also result in a reduced effectiveness of genotypic screening methods such as HMA.

ACKNOWLEDGEMENTS

The authors would like to thank Drs. Nirun Wanprapar, Kulkunya Chokephaibulkit and Sanay Chearskul, Department of Pediatrics, Faculty of Medicine Siriraj Hospital, Mahidol University and Drs. Piyaporn Bowonkiratikachorn and Kanittha Chimabutra, Charoenkrung-Pracharak Hospital for clinical specimens. The authors are indebted to Prof. James I Mullins, Dr. Gert Van der Auwera, Dr. Leo Heyndrickx and Assoc. Prof. Dr. Suda Louisirirotchanakul for their kindnesses and valuable advices about the HMA technique. The authors also thank the NIH AIDS Research and Reference Reagent Program, National Institutes of Health for providing the amplified reference plasmids in HMA and Dr. Janet McNicholl, the Thailand MOPH-US CDC Collaboration, A joint activity of the Thailand Ministry of Public Health & the U.S. Centers for Disease Control and Prevention for providing peptides and reagents in PEIA. This study was financially supported by the National HIV Repository and Bioinformatic Center (NHBRC), Thailand, Department of Microbiology, Faculty of Medicine Siriraj Hospital, and Ministry of Education and Faculty of Graduate Studies, Mahidol University, in the Academic Year 2000-2003.

REFERENCES

1. Myers G, Korber B, Wain-Hobson S, Jeang KT, Pavlakis GN. Human Retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, New Mexico, 1994.
2. Myers G, Korber B, Hahn B, Jeang KT, Mellors J, McCutchan F, Henderson L, Pavlakis GN. Human Retroviruses and AIDS. Los Alamos National Laboratory, Los Alamos, New Mexico, 1995.
3. Heyndrickx L, Janssens W, Zekeng L, *et al*. Simplified strategy for detection of recombinant human immunodeficiency virus type 1 group M isolates by gag/env heteroduplex mobility assay. Study Group on Heterogeneity of HIV Epidemics in African Cities. J Virol 2000; 74: 363-70.
4. Gao F, Robertson DL, Morrison SG, *et al*. The heterosexual human immunodeficiency virus type 1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. J Virol 1996; 70: 7013-29.
5. McCutchan FE, Hegerich PA, Brennan TP, *et al*. Genetic variants of HIV-1 in Thailand. AIDS Res Hum Retroviruses 1992; 8: 1887-95.
6. Wasi C, Herring B, Raktham S, *et al*. Determination of HIV-1 subtypes in injecting drug users in Bangkok, Thailand, using peptide-binding enzyme immunoassay and heteroduplex mobility assay: evidence of increasing infection with HIV-1 subtype E. AIDS 1995; 9: 843-9.
7. Artenstein AW, VanCott TC, Mascola JR, *et al*. Dual infection with human immunodeficiency virus type 1 of distinct envelope subtypes in humans. J Infect Dis 1995; 171: 805-10.
8. Tovanabutra S, Polonis V, De Souza M, *et al*. First CRF01_AE/B recombinant of HIV-1 is found in Thailand. AIDS 2001; 15: 1063-5.
9. Tovanabutra S, Beyrer C, Sakkhachornphop S, *et al*. The changing molecular epidemiology of HIV type 1 among northern Thai drug users, 1999 to 2002. AIDS Res Hum Retroviruses 2004; 20: 465-75.
10. Watanaveeradej V, DeSouza MS, Benenson MW, Sirisopana N, Nitayaphan S, Chanbancherd P, Brown AE, Sanders-Buell E, Birx DL, McCutchan FE, Carr JK. Subtype C/CRF01_AE recombinant HIV-1 found in Thailand. AIDS. 2003; 17: 2138-40.
11. Cham F, Heyndrickx L, Janssens W, *et al*. Development of a one-tube multiplex reverse transcriptase-polymerase chain reaction assay for the simultaneous amplification of HIV type 1 group M *gag* and *env* heteroduplex mobility assay fragments. AIDS Res Hum Retroviruses 2000; 16: 1503-5.
12. Delwart EL, Shpaer EG, Louwagie J, *et al*. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science 1993; 262: 1257-61.
13. Pau CP, Lee-Thomas S, Auwanit W, *et al*. Highly specific V3 peptide enzyme immunoassay for serotyping HIV-1 specimens from Thailand. AIDS 1993; 7: 337-40.
14. Cheingsong-Popov R, Osmanov S, Pau CP, *et al*. Serotyping of HIV type 1 infections: definition, relationship to viral genetic subtypes, and assay evaluation. UNAIDS Network for HIV-1 Isolation and Characterization. AIDS Res Hum Retroviruses 1998; 14: 311-8.
15. Murphy G, Belda FJ, Pau CP, *et al*. Discrimination of subtype B and non-subtype B strains of human immunodeficiency virus type 1 by serotyping: correlation with genotyping. J Clin Microbiol 1999; 37: 1356-60.
16. Vongsheree S, Phutiprawan T, Sri-ngam P, *et al*. Coexistence of HIV-1 subtypes B' and E infections among Thai injecting drug users. Asian Pac J Allergy Immunol 2002; 20: 29-35.
17. Kositanont U, Wasi C, Wanprapar N, *et al*. Primary infection of human herpesvirus 6 in children with vertical infection of human immunodeficiency virus type 1. J Infect Dis 1999; 180: 50-55.
18. Kunanusont C, Foy HM, Kreiss JK, *et al*. HIV-1 subtypes and male-to-female transmission in Thailand. Lancet 1995; 345: 1078-83.
19. Weniger BG, Takebe Y, Ou CY, *et al*. The molecular epidemiology of HIV in Asia. AIDS 1994; 8 Suppl 2: S13-28.
20. Hudgens MG, Longini IM, Jr., Vanichseni S, *et al*. Subtype-specific transmission probabilities for human immunodeficiency virus type 1 among injecting drug users in Bangkok, Thailand. Am J Epidemiol 2002; 155: 159-68.

21. Simon F, Loussert-Ajaka I, Damond F, *et al*. HIV type 1 diversity in northern Paris, France. AIDS Res Hum Retroviruses 1996; 12: 1427-33.
22. Menu E, Truong TX, Lafon ME, *et al*. HIV type 1 Thai subtype E is predominant in South Vietnam. AIDS Res Hum Retroviruses 1996; 12: 629-33.
23. Bobkov A, Kazennova E, Selimova L, *et al*. HIV type 1 *gag* D/*env* G recombinants in Russia. AIDS Res Hum Retroviruses 1998; 14: 1597-9.
24. Gaywee J, Artenstein AW, VanCott TC, *et al*. Correlation of genetic and serologic approaches to HIV-1 subtyping in Thailand. J Acquir Immune Defic Syndr Hum Retrovirol 1996; 13: 392-6.
25. Peeters M, Nkengasong J, Willems B, *et al*. Antibodies to V3 loop peptides derived from chimpanzee lentiviruses and the divergent HIV-1ANT-70 isolate in human sera from different geographic regions. AIDS 1994; 8: 1657-61.
26. Massanga M, Ndoyo J, Hu DJ, *et al*. A highly heterogeneous HIV-1 epidemic in the Central African Republic. Emerg Infect Dis 1996; 2: 222-4.
27. Ou CY, Takebe Y, Weniger BG, Luo CC, *et al*. Independent introduction of two major HIV-1 genotypes into distinct high-risk populations in Thailand. Lancet 1993; 341: 1171-4.
28. Dickover RE, Garratty EM, Plaeger S, *et al*. Perinatal transmission of major, minor, and multiple maternal human immunodeficiency virus type 1 variants *in utero* and intrapartum. J Virol 2001; 75: 2194-203.
29. van der Groen G, Nyambi PN, Beirnaert E, *et al*. Genetic variation of HIV type 1: relevance of interclade variation to vaccine development. AIDS Res Hum Retroviruses 1998; 14 Suppl 3: S211-21.
30. Nkengasong JN, Willems B, Janssens W, *et al*. Lack of correlation between V3-loop peptide enzyme immunoassay serologic subtyping and genetic sequencing. AIDS 1998; 12: 1405-12.